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PCT/NZ03/00184

CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 17 March 2003 with an application for Letters Patent number 524796 made by PROTEMIX CORPORATION LIMITED.

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Dated 11 September 2003.

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524796

NEW ZEALAND

PATENTS ACT, 1953

PROVISIONAL SPECIFICATION

"Dosage Forms and Related Therapies"

We, PROTEMIX CORPORATION LIMITED, a company duly incorporated under the laws of New Zealand of Level 4, 41 Shortland Street, Auckland, New Zealand, do hereby declare this invention to be described in the following statement:

The present invention relates to (particularly diabetic) heart and coronary disease therapies and more particularly to methods of

(A) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extracellular space and increased deposition of extracellular matrix (and its consequences) and/or coronary artery structure damage selected from at least media damage (the muscle layer) and intima damage (the endothelial layer) (and its consequences), and/or

(B) improving in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") any one or more of systolic function, diastolic function, contractility, recoil characteristics and ejection fraction (e.g. as determined clinically, by ultrasound, MRI or other imagining, and/or

(C) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") disorders or the heart muscle, macrovascular disease, microvascular disease and plaque rupture of athereomatous lesions of major blood vessels (and consequences thereof)

reliant upon, as active ingredient(s), trientine (See Martindale 33rd edition, 1025.3), salts of trientine and/or metabolites thereof.

The invention also consists in methods of reversing in a patient at least some of any damage arising from diabetic kidney disease, diabetic nephropathy and/or copper accumulation in the kidney, and/or reversing in a patient at least some of any damage to the renal arteries reliant upon the abovementioned active ingredient(s).

The patient may have elevated copper levels.

Reference to "copper levels capable of diminishment" may mean elevated copper levels but not necessarily so. The term "capable of diminishment" refers to copper values

readily scavenged by the trientine chelating moiety as opposed, we believe, to those copper values less readily scavenged (likely non interstitially located copper values).

Included in the present invention are dosage forms of the active ingredient(s) and relates to related uses (including uses in the preparation of pharmaceutical compositions of trientine, salts of trientine and/or metabolites thereof). Such dosage forms are indicated for at least both diabetic and elevated copper level patients.

By way of background the following can be stated in respect of diabetic heart disease in the human being,

1. Worldwide prevalence of diabetes is increasing. Number of cases of type 2 diabetes projected to increase from 135 million in 2000 to more than 300 million in 2025. Increase is related to ageing of the population, increasing obesity, and low socio-economic status. See, WHO. The World Health Report 1997.

2. Mortality from diabetes has increased over the last decade whereas mortality from cardiovascular disease, stroke, and malignant diseases has remained static or declined. See, US Centre for Health Studies.

3. Causes of premature mortality in type 2 diabetes comprise cardiovascular disease, 58%; cerebrovascular disease, 12%; nephropathy, 3%; diabetic coma, 1%; malignancy, 11%; and infections 4%. See, Pickup J, Williams G eds. Handbook of diabetes, 2nd edition, 1999; p 24.

4. Diabetic heart disease is characterised by more severe coronary artery disease at a younger age, a 4-fold increased frequency of heart failure post-acute myocardial infarction and a disproportionate increase in left ventricular hypertrophy. See Struthers AD, Morris Ad, Lancet 2002;359:1430-2.

5. Patients with type 2 diabetes manifest a disproportionate increase in mortality within the first 24-hours post-acute myocardial infarction. Acute intervention can ameliorate this risk. See, Malmberg K Br Med J 1997;314:1512-5.

Much of the above is equally applicable to diabetic coronary artery structure.

PCT/NZ99/00161 (published as WO00/18392 on 6 April 2000) has disclosed a method of treating a mammalian patient predisposed to and/or suffering from diabetes mellitus with a view to minimising the consequences of macrovascular and microvascular

damage to the patent which comprises, in addition to any treatment in order to control blood glucose levels, at least periodically inhibiting or antagonizing fructosamine oxidase enzyme activity in the patient. An assay for such activity is disclosed in their PCT/NZ99/00160 (published as WO00/18891 on 6 April 2000).

A range of different agents capable of acting as fructosamine oxidase inhibitors and/or antagonists were disclosed in PCT/NZ99/00161. These included copper chelating agents, substrate analogues and hydrazine compounds.

The full contents of the aforementioned specifications are here included by way of reference.

We have hypothesised that reduction in available free copper does have an affect in preventing macrovascular, microvascular and/or toxic/metabolic diseases of the kind hereinafter exemplified and in tissue repair processes. This is irrespective of the glucose metabolism of the patient.

We have also hypothesized that cardiovascular accumulation of redox-active transition metal ions is responsible for many of the adverse outcomes in diabetes. Under physiological conditions, injury to a target organ is sensed by distant stem cells, which migrate to the site of damage then undergo alternate stem cell differentiation; these events promote structural and functional repair. However, the accumulation of redox-active transition metals, particularly copper in cardiac or vascular tissues in subjects with diabetes is accompanied by a suppression of the normal tissue regeneration effected by the migration of stem cells. Elevated tissue levels of copper suppress these normal biological behaviours of such undifferentiated cells. Conditions occurring in the context of diabetes or impaired glucose tolerance, in which the suppression of normal stem cell responses can cause impairment of normal tissue responses, include the following:

1. Cardiac failure
2. Acute myocardial infarction
3. Wound healing and ulceration
4. Tissue damage caused by infection
5. Diabetic kidney damage

Conditions in which therapy to lower copper values in diabetic patients (ie; with IGT or Type 2 Diabetes Mellitus) is liable to prove beneficial include at least the following:

1. HEART FAILURE IN THE CONTEXT OF DIABETES

Significant regeneration of cardiac tissues can occur within a few days of cardiac transplantation. The likely mechanism is migration of stem cells from extra-cardiac sites to the heart, with subsequent differentiation of such cells into various specialized cardiac cells, including myocardial, endothelial and coronary vascular cells. We believe that copper accumulation in cardiac tissues is likely to severely impair these regenerative responses. Hence a role for acute intravenous therapy with a copper chelator in the treatment of diabetic heart failure.

2. MYOCARDIAL INFARCTION IN THE CONTEXT OF DIABETES.

Myocardial infarction is accompanied by proliferation of cells in the ventricular myocardium when MI occurs in the context of diabetes, the presence of elevated tissue levels of redox-active transition metals suppresses normal stem cell responses, resulting in impaired structural and functional repair of damaged tissues. Up to 20% of cells in the heart may be replaced by stem cell migration from extra-ventricular sites, as soon as four days after cardiac transplantation. These observations suggest that treatment of AMI in the context of diabetes will be improved by acute (if necessary, parenteral) as well as by subsequent chronic administration of chelators. The mechanism of the impairment of cardiac function in diabetes is likely a toxic effect of accumulated transition metals on tissue dynamics, resulting in impaired tissue regeneration caused in turn by suppression of normal stem cell responses, which mediate physiological tissue regeneration by migration to damaged tissue from external sites.

3. WOUND HEALING AND ULCERATION IN THE CONTEXT OF DIABETES

The processes of normal tissue repair require intervention of mobilizing stem cells, which effect repair of the various layers of blood vessels, for example. We believe that an accumulation of transition metals (particularly copper) in vascular tissues causes the

impaired tissue behaviour characteristic of diabetes, including impaired wound repair following surgery or trauma, and the exaggerated tendency to ulceration and poor healing of established ulcers. We believe that the treatment of diabetics with copper chelators before they undergo surgery, or in the context of traumatic tissue damage, is likely to be of benefit. It is probable that surgery in diabetics would have a better outcome if excess transition metals were removed from blood vessels prior to surgery. This may need to be accomplished on either an acute basis (with parenteral therapy) or on a more chronic basis (with oral therapy) prior to actual surgery.

4. SOFT TISSUE DAMAGE RESULTING FROM INFECTION AND OCCURING IN THE CONTEXT OF DIABETES OR IMPAIRED GLUCOSE TOLERANCE

We believe the processes of normal tissue repair following infection require intervention of mobilized stem cells, which migrate to sites of tissue damage to effect tissue regeneration and repair, for example, of the various layers of blood vessels. Such tissue damage will be impaired by suppressed stem cell responses, such as those caused by the build up of redox-active transition metals (particularly copper) in tissues, for examples the walls of blood vessels.

5. KIDNEY DAMAGE OCCURING IN THE CONTEXT OF DIABETES

We believe that impaired stem cell responses in the kidneys of diabetics contribute to diabetic nephropathy and renal failure. We believe that treatment of diabetics having kidney failure by administration of a copper chelator will improve organ regeneration by restoring normal tissue healing by allowing stem cells to migrate and differentiate normally.

However, even in the non diabetic mammal and even in a mammal without a glucose mechanism abnormality, we have hypothesized a reduction in extra-cellular copper values is advantageous in that such lower levels will lead to one or both a reduction in copper mediated tissue damage and improved tissue repair by restoration of normal tissue stem cell responses.

In our own studies (using the streptozocin-diabetic (STZ) rat model) we have found a high frequency of tissue damage in the heart tissue and coronary artery tissue in severely diabetic animals. This reflects what is found in man.

We now more firmly take the view that copper values (and particularly copper II) not bound internally of cells is available to mediate (together with available reducing substances) the generation of damaging free radicals that have a role in both tissue damage and impairment of stem cell mediated repair of such tissue. This is irrespective of diabetic status but we believe is more prevalent in diabetic rats and other mammals including human beings.

In respect of such damage and repair impairment we propose a diminishment in available free copper values as being an appropriate preventive and/or treatment approach for diabetic patients or any patient (particularly a patient not suffering Wilson Disease) who has elevated copper levels.

Our agent of choice is trientine, preferably as an acid addition salt.

Alternative names for trientine include *N,N'-Bis(2-aminoethyl)-1,2-ethanedi-amine; triethylenetetramine;* 1,8-diamino-3,6-diazaoctane; 3,6-diazaoctane-1,8-diamine; 1,4,7,10-tetraazadecane; trien; TETA; TECZA and triene.

Reference made herein to "trientine" refers to the moiety substantially of the structure depicted but can include analogues thereof which are prodrugs of the active copper chelating moiety or metabolite of trientine. Salts of trientine (which optionally can be salts of a prodrug of the trientine copper chelating moiety or metabolite) are preferably acid addition salts such as, for example, those of suitable mineral or organic acids, eg; the hydrochlorides, maleates, citrates, tartrates, etc.

Salts of trientine (such as acid addition salts, eg; trientine dihydrochloride) act as copper-chelating agents, which aids the elimination of copper from the body by forming a stable soluble complex that is readily excreted by the kidney.

Trientine, a strongly basic moiety, with its multiple nitrogens can be converted into a large number of suitable associated acid addition salts using an acid, for example, by reaction of stoichiometrically equivalent amounts of trietine and of the acid in an inert solvent such as ethanol or water and subsequent evaporation if the dosage form is best

formulated from a dry salt. Possible acids for this reaction are in particular those which yield physiologically acceptable salts. Thus inorganic acids can be used, e.g. sulfuric acid, nitric acid, hydrohalic acids such as hydrochloric acid or hydrobromic acid, phosphoric acids such as orthophosphoric acid, sulfamic acid. Furthermore organic acids, can be used, in particular aliphatic, alicyclic, araliphatic, aromatic or heterocyclic mono- or polybasic carboxylic, sulfonic or sulfuric acids, (e.g. formic acid, acetic acid, propionic acid, pivalic acid, diethylacetic acid, malonic acid, succinic acid, pimelic acid, fumaric acid, maleic acid, lactic acid, tartaric acid, malic acid, citric acid, gluconic acid, ascorbic acid, nicotinic acid, isonicotinic acid, methane-or ethanesulfonic acid, ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, naphthalenemono-and-disulfonic acids and laurylsulfuric acid).

The trientine moieties can also be in the form of quarternary ammonium salts in which the nitrogen atom carries a suitable organic group such as an alkyl, alkenyl, alkynyl or aralkyl moiety.

Preferably the trientine moieties are in the form of a compound or buffered in solution and/or suspension to a near neutral pH much lower than the pH of 14 of trientine itself.

Suitable anions may include citrate, isocitrate, α-Ketoglutarate, Succinate, Fumarate, Malate, Oxaloacetate, Acetate and pyruvate.

Trientine moieties (preferably delivered as a salts of trientine (such as acid addition salts, eg; trientine dihydrochloride) act as copper-chelating agents, which aids the elimination of copper from the body by forming a stable soluble complex that is readily excreted by the kidney.

The presumed site of action of the chelating trientine moiety of a salt such as trientine dihydrochloride is the removal of loosely bound copper from the body and in particular from the cardiac extracellular matrix and the coronary extracellular matrix.

Bioavailabilities of the active species of trientine dihydrochloride after oral administration is low (<10%) due to poor absorption and marked first-pass metabolism. Trientine dihydrochloride and its transformed metabolite, *N*-acetyl-trientine hydrochloride, are both capable of binding copper, although the chelating activity of the

analogue *N*-acetyl-trientine hydrochloride is significantly lower than trientine dihydrochloride. See, Kodama H. *Life Sciences* 1997;61:899-907.

Absorption of trientine dihydrochloride is adversely affected by food, mineral supplements and other drugs.

We have now shown in the STZ rat model for both diabetic and non diabetic man a reduction in available free copper does have an affect in reversing in the diabetic rat both (i) cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extra cellular space and increased deposition of extra cellular matrix (and its consequences) and (ii) coronary artery structure damage (and its consequences). We have also shown amelioration. In so showing reversal of damage in the STZ, we have found a dose relativity for man insofar as the copper scavenging into the urine is concerned.

Under physiological conditions we believe injury to the cardiac structure is sensed by distant stem cells, which migrate to the site of damage then undergo alternate stem cell differentiation; these events promote structural and functional repair. However, the accumulation of redox-active transition metals, particularly copper in cardiac tissues and coronary arteries in subjects with diabetes we believe is accompanied by a suppression of the normal tissue regeneration effected by the migration of stem cells. Elevated tissue levels of copper suppress these normal biological behaviours of such undifferentiated cells.

Even in the non diabetic mammal (e.g. without Type 2 Diabetes mellitus) and even in a mammal without a glucose mechanism abnormality (e.g. without IGT), we believe a reduction in extra-cellular copper values is advantageous in that such lower levels will lead to one or both a reduction in copper mediated tissue damage and improved tissue repair by restoration of normal tissue stem cell responses.

It is an object of the present invention to provide sustained, controlled and/or extended release dosage forms useful for taking advantage of this prospect for the purpose of amelioration of such structure damage and damage reversal all irrespective of whether or not our hypothesis or proposals as to mode of action are correct.

Such damage includes cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extracellular space and increased deposition of extracellular matrix (and its consequences) and/or coronary artery structure damage selected from at least media damage (the muscle layer) and intima damage (the endothelial layer) (and its consequences), whether or not the hypotheses or proposals as to mode of action are correct.

It is another object to provide uses and dosage forms applicable instead or as well to improve in a human being or other mammal (preferably diabetic and/or with a raised copper level) ("the patient") any one or more of systolic function, diastolic function, contractility, recoil characteristics and ejection fraction (e.g. as determined clinically, by ultrasound, MRI or other imaging), whether or not the hypotheses or proposals as to mode of action are correct.

It is another object of the present invention to provide methods of treatment and related methods, uses and pharmaceutical compositions that ameliorate, prevent or treat any one or more disease states of the cardiovascular tree (including the heart) and dependent organs (eg; retina, kidney, nerves, etc.) exacerbated by elevated non-intracellar free copper values levels, whether or not the hypotheses or proposals as to mode of action are correct.

Reference herein to diseases of the cardiovascular tree and diseases of dependent organs includes any one or more of

(i) **disorders of the heart muscle** (cardiomyopathy or myocarditis) such as idiopathic cardiomyopathy, metabolic cardiomyopathy which includes diabetic cardiomyopathy, alcoholic cardiomyopathy, drug-induced cardiomyopathy, ischemic cardiomyopathy, and hypertensive cardiomyopathy,or

(ii) atheromatous disorders of the major blood vessels (**macrovascular disease**) such as the aorta, the coronary arteries, the carotid arteries, the cerebrovascular arteries, the renal arteries, the iliac arteries, the femoral arteries, and the popliteal arteries,or

(iii) toxic, drug-induced, and metabolic (including hypertensive and/or diabetic disorders of small blood vessels (**microvascular disease**) such as the retinal arterioles, the glomerular arterioles, the vasa nervorum, cardiac arterioles, and associated capillary beds of the eye, the kidney, the heart, and the central and peripheral nervous systems,or

(iv) **plaque rupture of atheromatous lesions of major blood vessels** such as the aorta, the coronary arteries, the carotid arteries, the cerebrovascular arteries, the renal arteries, the iliac arteries, the fermoral arteries and the popliteal arteries.

The present invention relates to any such ailments and their treatment irrespective (unless otherwise stated) of any diabetic and/or glucose abnormality state of the mammalian patient.

Compliance of a dosage regime is always essential in order to derive the best benefit from a treatment regime. The present invention recognises a benefit from sustained release dosage forms that can provide such levels of sustained delivery to a patient as are required to elicit the advantages now seen from the prospect of lower overall dose delivery of trientine formulations when one compares them to the twice a day multiple dosage oral regimes hitherto used with trientine formulations for Wilson's disease.

The present invention in one aspect consists in **a parenteral formulation or dosage form** capable of delivery of an effective amount of trientine hydrochloride and/or its metabolites when administered or self administered to a human being or other mammal (preferably diabetic or predisposed thereto or with elevated copper levels) sufficient

(A) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extracellular space and increased deposition of extracellular matrix (and its consequences), and/or coronary artery structure damage (and its consequences),

(B) improving in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient")

any one or more systolic function, diastolic function, contractility, recoil characteristics and ejection fraction (e.g. as determined clinically, by ultrasound, MRI or other imaging),

(C) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") disorders of the heart muscle, macrovascular disease, microvascular disease and plaque rupture of athereomatous lesions of major blood vessels (and the consequences thereof), and/or

(D) reversal of at least some of any damage arising from diabetic kidney disease, diabetic nephropathy and/or copper accumulation in the kidney and/or reversal of at least some of any damage to the renal arteries.

In certain circumstances, oral administration of trientine is not possible or desirable. For example, acute myocardial infarction is often accompanied by nausea and vomiting, rendering the oral route of administration ineffective. Gastric emptying may also be delayed under these conditions. There is thus a need for a parenteral (eg; an injectable) composition containing trientine or a pharmaceutically acceptable salt thereof at least for the treatment of patients with acute coronary syndrome.

Suitable parenteral forms include solutions, suspensions, emulsions etc. that can be administered parenterally by either subcutaneous injections, intravenous, intramuscular, intradermal, intrastemal injection or infusion techniques.

Accordingly in another aspect the present invention in one aspect consists in a **method** of

(A) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extracellular space and increased deposition of extracellular matrix (and its consequences), and/or coronary artery structure damage (and its consequences),

(B) improving in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient")

any one or more systolic function, diastolic function, contractility, recoil characteristics and ejection fraction (e.g. as determined clinically, by ultrasound, MRI or other imaging),

(C) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") disorders of the heart muscle, macrovascular disease, microvascular disease and plaque rupture of athereomatous lesions of major blood vessels (and the consequences thereof), and/or

(D) reversal of at least some of any damage arising from diabetic kidney disease, diabetic nephropathy and/or copper accumulation in the kidney and/or reversal of at least some of any damage to the renal arteries.

which method comprises or includes the step of administration and/or self administration to the patient an effective amount of a parenteral formulation or dosage form, said formulation or dosage form having as the or an active agent a suitable trientine moiety (e.g. trientine, at least one salt of trientine and/or at least one metabolite of trientine and/or its salt(s) ("trientine" including analogues thereof and/or prodrugs thereof)).

Preferably the effective amount is sufficient to provide effective chelation of copper for an overall diminishment thereof in the patient.

Preferably the effective amount is of trientine dihydrochloride.

In a further aspect the present invention consists in **the use** of a suitable trientine moiety (e.g. trientine, at least one salt of trientine and/or at least one metabolite of trientine and/or its salt(s) (the "active agent(s)")), together with other material(s) appropriate for a parenteral formulation or dosage form, **in the manufacture of a parenteral formulation or dosage form** useful for

(A) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") cardiac structure damage selected from one or more of atrophy, loss of myocytes, expansion of the extracellular space and increased deposition of extracellular matrix (and its consequences), and/or coronary artery structure damage (and its consequences),

(B) improving in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") any one or more systolic function, diastolic function, contractility, recoil characteristics and ejection fraction (e.g. as determined clinically, by ultrasound, MRI or other imaging),

(C) ameliorating and/or reversing in (I) a diabetic human being or other diabetic mammal or (II) a human being or other mammal with copper levels capable of diminishment ("the patient") disorders of the heart muscle, macrovascular disease, microvascular disease and plaque rupture of athereomatous lesions of major blood vessels (and the consequences thereof), and/or

(D) reversal of at least some of any damage arising from diabetic kidney disease, diabetic nephropathy and/or copper accumulation in the kidney and/or reversal of at least some of any damage to the renal arteries.

We have determined in our trials referred to hereinafter that a divided dose of 1.2 g/day is effective for and yet (insofar as an instantaneous body level is concerned) in excess of dosage levels to be required chronically in practice for the purpose of amelioration and/or reversal of cardiac structure damage and/or coronary artery structure damage. Such a dose rate of 1.2 g/day is capable of being provided parenterally.

In another aspect the present invention consists in a method of administering an effective amount of Trientine formulated in a parenterally acceptable formulation.

Preferably said formulation is suitable for use in the treatment of any of heart failure, diabetic heart disease, acute coronary syndrome, hypertensive heart disease, ischaemic heart disease, coronary artery disease, peripheral arterial disease, Wilson's disease, or any form of cancer.

Preferably said formulation contains an effective dosage unit to the patient of the trientine from 1 mg to 600mg per uniit.

Preferably the total daily dose rate is from between 5gms to 1mg.

Preferably the dosage unit will maintain a constant blood plasma concentration from between 1 hour to 24 hour.

In another aspect of the present invention consists in a formulation of trientine that maintains constant plasma concentrations of the drug for extended periods and is effective in removing copper from the body of patients with any of heart failure, diabetic heart disease, acute coronary syndrome, hypertensive heart disease, ischaemic heart disease, coronary artery disease, peripheral arterial disease, Wilson's disease, or any form of cancer.

Reference herein to "elevated" in relation to the presence of copper values will include humans having at least 10 mcg free copper/dL of serum when measured as discussed by Merck & Co Inc below.

A measurement of free copper [which equals total plasma copper minus ceruloplasmin-bound copper] can be made using the procedure disclosed in the Merck & Co Inc datasheet (www.Merck.com) for SYPRINE® (trientine dihydrochloride) capsules where they state in respect of the use of trientine dihydrochloride for the copper values excesses of Wilson's Disease:

"The most reliable index for monitoring treatment is the determination of free copper in the serum, which equals the difference between quantitatively determined total copper and ceruloplasmin-copper. Adequately treated patients will usually have less than 10 mcg free copper/dL of serum.

Therapy may be monitored with a 24 hour urinary copper analysis periodically (i.e. every 6-12 months). Urine must be collected in copper-free glassware. Since a low copper diet should keep copper absorption down to less than one milligram a day, the patient probably will be in the desired state of negative copper balance if 0.5 to 1.0 milligram of copper is present in a 24-hour collection of urine"

We have conducted studies reliant on trientine dihydrochloride in the STZ rat model as well in humans and wish to describe the invention further by reference to the accompanying drawings in which:

Figure 1 is a diagram showing various pathways addressed by the present invention.

Figure 2 is a hypothesis of the mechanisms involved applicable to cardiomyopathy and macrovascular disease in a patient with type 2 diabetes or impaired glucose tolerance, for example, such a hypothesis showing reliance on a possible fructosamine oxidase/superoxide dismutase generation of a precursor to an copper catalyzed reaction (the Haber-Weiss Reaction) which generates the harmful free radicals.

Figure 3 is the methodology for a human patient with suspected cardiomyopathy under the present invention.

Figure 4 is a similar diagram to that of Figure 3 but in respect of a patient with suspected macrovascular disease.

Figure 5 is a diagram showing the body weight of animals changing over the time period of experiment.

Figure 6 shows the glucose levels of the animals changing over the time period of the experiment.

Figure 7 is a diagram showing cardiac output.

Figure 8 is a diagram showing coronary flow.

Figure 9 is a diagram showing coronary flow normalized to final cardiac weight.

Figure 10 is a diagram showing aortic flow.

Figure 11 is a diagram showing the maximum rate of positive change in pressure development in the ventricle with each cardiac cycle (contraction).

Figure 12 is a diagram showing the maximum rate of decrease in pressure in the ventricle with each cardiac cycle (relaxation).

Figure 13 shows the percentage of functional surviving hearts at each after-load. **Figure 14** shows diagrammatically how the extracted heart was attached to the modified apparatus.

Figure 15 shows diagrammatically the heart depicted in Figure 14 in more detail (picture adapted from Grupp I *et al.*, *Am J Physiol* 34:H1401-1410 (1993)).

Figure 16 shows the urine excretion in diabetic and non diabetic animals in response to increasing doses of trientine or equivalent volume of saline, wherein urine excretion in diabetic and nondiabetic animals in response to increasing doses of trientine (*bottom;* 0.1, 1.0, 10, 100 $mg.kg^{-1}$ in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or an equivalent volume of saline *(top)*, and each point represents a 15 min urine collection period (see Methods for details); error bars show SEM and P values are stated if significant ($P < 0.05$).

Figure 17 shows urine excretion in non diabetic and diabetic animals receiving increasing doses of trientine or an equivalent volume of saline, wherein urine excretion in diabetic *(top)* and nondiabetic *(bottom)* rats receiving increasing doses of trientine (0.1, 1.0, 10, 100 $mg.kg^{-1}$ in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or an equivalent volume of saline, and each point represents a 15 min urine collection period (see Methods for details); error bars show SEM and P values are stated if significant ($P < 0.05$).

Figure 18 shows copper excretion in the urine of diabetic and non diabetic animals receiving increasing doses of trientine or an equivalent volume of saline, wherein copper excretion in urine of diabetic *(top)* and nondiabetic *(bottom)* rats receiving increasing doses of trientine (0.1, 1.0, 10, 100 $mg.kg^{-1}$ in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or an equivalent volume of saline, and each point represents a 15 min urine collection period (see Methods for details); error bars show SEM and P values are stated if significant ($P < 0.05$).

Figure 19 shows the same information in Figure 18 with presentation of urinary copper excretion per gram of bodyweight, wherein urinary copper excretion per gram of bodyweight in diabetic and nondiabetic animals in response to increasing doses of trientine (*bottom;* 0.1, 1.0, 10, 100 $mg.kg^{-1}$ in 75 µl saline followed by 125 µl saline flush injected at time shown by arrow) or

an equivalent volume of saline *(top)*, and each point represents a 15 min urine collection period (see Methods for details); error bars show SEM and P values are stated if significant ($P < 0.05$).

Figure 20 shows the total amount of copper excreted in non diabetic and diabetic animals administered saline or drug, wherein total urinary copper excretion (μmol) in nondiabetic animals administered saline (black bar, n = 7) or trientine (hatched bar, n = 7) and in diabetic animals administered saline (grey bar, n = 7) or trientine (white bar, n = 7); error bars show SEM and P values are stated if significant ($P < 0.05$).

Figure 21 shows the total amount of copper excreted per gram of bodyweight in animals receiving trientine or saline, wherein total urinary copper excretion per gram of bodyweight ($\mu g.gBW^{-1}$) in animals receiving trientine (nondiabetic: hatched bar, n = 7; diabetic: white bar, n = 7) or saline (nondiabetic: black bar, n = 7; diabetic: grey bar, n = 7); error bars show SEM and P values are stated if significant ($P < 0.05$).

Figure 22 shows the iron excretion in urine of diabetic and non diabetic animals receiving increasing doses of trientine or an equivalent volume of saline, wherein iron excretion in urine of diabetic *(top)* and nondiabetic *(bottom)* rats receiving increasing doses of trientine (0.1, 1.0, 10, 100 $mg.kg^{-1}$ in 75 μl saline followed by 125 μl saline flush injected at time shown by arrow) or an equivalent volume of saline, and each point represents a 15 min urine collection period (see Methods for details); error bars show SEM and P values are stated if significant ($P < 0.05$).

Figure 23 shows the urinary iron excretion per gram of bodyweight in diabetic and non diabetic animals receiving trientine or saline, wherein urinary iron excretion per gram of bodyweight in diabetic and nondiabetic animals in response to increasing doses of trientine (*bottom;* 0.1, 1.0, 10, 100 $mg.kg^{-1}$ in 75 μl saline followed by 125 μl saline flush injected at time shown by arrow) or an equivalent volume of saline *(top)*, and each point represents a 15 min

urine collection period (see Methods for details); error bars show SEM and *P* values are stated if significant ($P < 0.05$).

Figure 24 shows the total urinary iron excretion in non diabetic and diabetic animals administered saline or drug, wherein total urinary iron excretion (μmol) in nondiabetic animals administered saline (black bar, n = 7) or trientine (hatched bar, n = 7) and in diabetic animals administered saline (grey bar, n = 7) or trientine (white bar, n = 7); error bars show SEM and *P* values are stated if significant ($P < 0.05$).

Figure 25 shows the total urinary iron excretion per gram of bodyweight in animals receiving trientine or saline, wherein Total urinary iron excretion per gram of bodyweight ($\mu g.gBW^{-1}$) in animals receiving trientine (nondiabetic: hatched bar, n = 7; diabetic: white bar, n = 7) or saline (nondiabetic: black bar, n = 7; diabetic: grey bar, n = 7); error bars show SEM and *P* values are stated if significant ($P \leq 0.05$).

Figure 26 shows the percentage of surviving hearts at each after-load pressure.

Figure 27 is a table comparing the copper and iron excretion in the animals receiving trientine or saline, which is a statistical analysis using a mixed linear model.

Figure 28 shows urinary [Cu] by AAS (△) and EPR (▲) following sequential 10 $mg.kg^{-1}$ (A) and 100 (B) trientine boluses, as in Figure 19; (*inset*) background-corrected EPR signal from 75-min urine indicating presence of Cu^{II}-trientine; *, $P < 0.05$, **, $P < 0.01$ vs. control.

Figure 29 shows the structure of LV-myocardium from STZ-diabetic and matched non-diabetic control rats following 7-w oral trientine treatment, wherein cardiac sections were cut following functional studies. Each image is representative of 5 independent sections per heart x 3 hearts per treatment. **a — d**, Laser confocal images of 120-μM LV sections co-stained for actin (Phalloidin-488, orange) and immunostained for β_1-integrin (CY5-conjugated secondary

antibody, purple) (scale-bar = 33 μm). **a**, Untreated-control; **b**, Untreated-diabetic; **c**, Trientine treated diabetic; **d**, Trientine-treated non-diabetic control. **e** — **h**, TEM images of corresponding 70-nM sections stained with uranyl acetate/lead citrate (scale-bar = 158 nm); **e**, Untreated-control; **f**, Untreated-diabetic; **g**, Trientine-treated diabetic; **h**, Trientine-treated non-diabetic control.

Figure 30 shows plasma concentration-time profiles of trientine after oral administration to four male patients.

Figure 31 shows plasma concentration-time profiles of trientine after oral administration to four female patients.

Figure 32 shows a randomised, double blind, placebo-controlled trial comparing effects of oral trientine and placebo on urinary Cu excretion from male humans with uncomplicated T2DM and matched non-diabetic controls, wherein urinary Cu excretion ($\mu mol.2\ h^{-1}$ on day 1 (baseline) and day 7 following a single 2.4-g oral dose of trientine or matched placebo to subjects described in Table 9, placebo- treated T2DM, ?, placebo-treated control, ?, trientine-treated T2DM, □; trientine treated control,■. Cu excretion from T2DM following trientine-treatment was significantly greater than that from trientine-treated non-diabetic controls ($P < 0.05$).

Figure 33 shows effect of 6 months' oral trientine treatment on LV mass in humans with T2DM, wherein trientine (1.2 g twice-daily) or matched placebo were administered to subjects with diabetes (n = 15) or matched controls (n = 15) in a double-blind, parallel-group study, and wherein differences in LV mass (g; mean and 95% confidence interval) were determined by tagged-cardiac MRI.

DETAILED DESCRIPTION OF THE INVENTION

The invention is related to and describes the methods relating to discoveries surrounding increased tissue copper and mechanisms leading to tissue damage, including nerve and vascular

damage, for example, diabetic nerve and/or vascular damage. It is believed, without wishing to be bound by any particular mechanism or theory of operation or effectiveness, that tissue accumulation of trace metals plays a role in the mechanisms of tissue damage in diabetes as well as in other disorders, diseases, and conditions as set forth or referenced or suggested herein. Histological evidence from experiments showed that six months of treatment with trientine appears to protect the hearts of diabetic Wistar rats from development of diabetic damage (cardiomyopathy) as judged by histology. The doses of trientine required for copper and iron to be excreted in the urine have also been investigated, for example, as well as possible differences between the excretion of these metals in diabetic and nondiabetic animals. For example, the excretion profiles of copper and iron in the urine of normal and diabetic rats were compared after acute intravenous administration of increasing doses of trientine. Additionally, it was ascertained whether acute intravenous administration of trientine has acute adverse cardiovascular side effects. Methods used in the experimentals were as follows.

Male Wistar rats (n = 28, 303 ± 2.9 g) were divided randomly into diabetic and nondiabetic groups. Following induction of anesthesia (5% halothane and 2l.min^{-1} O_2), animals in the diabetic group received a single intravenous dose of streptozotocin (STZ, 55mg.kg^{-1} body weight, Sigma; St. Louis, MO) in 0.5 ml saline administered via the tail vein. Nondiabetic animals received an equivalent volume of saline. Following injection, both diabetic and nondiabetic rats were housed in like-pairs and provided with access to normal rat chow (Diet 86 pellets; New Zealand Stock Feeds, Auckland, NZ) and deionized water *ad libitum*. Blood glucose and body weight were measure at day 3 following STZ/saline injection and then weekly throughout the study. Diabetes was identified by polydipsia, polyuria and hyperglycemia (> 11 mmol.l^{-1}, Advantage II, Roche Diagnostics, NZ Ltd).

Six to seven weeks (mean = 44 ± 1 days) after administration of STZ, animals underwent either a control or drug experimental protocol. All animals were fasted overnight prior to surgery but continued to have *ad libitum* access to deionized water. Induction and maintenance of surgical anesthesia was by 3 - 5% halothane and $2l.min^{-1}$ O_2. The femoral artery and vein were cannulated with a solid-state blood pressure transducer (Mikrotip™ 1.4F, Millar Instruments, Texas, USA) and a saline filled PE 50 catheter respectively. The ureters were exposed via a midline abdominal incision, cannulated using polyethylene catheters (external diameter 0.9mm, internal diameter 0.5mm) and the wound sutured closed. The trachea was cannulated and the animal ventilated at 70-80 $breaths.min^{-1}$ with air supplemented with O_2 (Pressure Controlled Ventilator, Kent Scientific, Connecticut, USA). The respiratory rate and end-tidal pressure (10-15 cmH_2O) were adjusted to maintain end-tidal CO_2 at 35-40 mm Hg (SC-300 CO_2 Monitor, Pryon Corporation, Wisconsin, USA). Body temperature was maintained at 37°C throughout surgery and the experiment by a heating pad. Estimated fluid loss was replaced with intravenous administration of 154 $mmol.l^{-1}$ NaCl solution at a rate of 5 $ml.kg^{-1}.h^{-1}$.

Following surgery and a 20 min stabilization period, the experimental protocol was started. Trientine was administered intravenously over 60 s in hourly doses of increasing concentration (0.1, 1.0, 10 and 100 $mg.kg^{-1}$ in 75 µl saline followed by 125 µl saline flush). Control animals received an equivalent volume of saline. Urine was collected in 15 min aliquots throughout the experiment in pre-weighed polyethylene epindorph tubes. At the end of the experiment a terminal blood sample was taken by cardiac puncture and the separated serum stored at -80°C until future analysis. Hearts were removed through a rapid mid-sternal thoracotomy and processed as described below.

Mean arterial pressure (MAP), heart rate (HR, derived from the MAP waveform) oxygen saturation (Nonin 8600V Pulse Oximeter, Nonin Medical Inc., Minnesota, USA) and core

body temperature, were all continuously monitored throughout the experiment using a PowerLab/16s data acquisition module (AD Instruments, Australia). Calibrated signals were displayed on screen and saved to disc as 2 s averages of each variable.

Instrumentation: A Perkin Elmer (PE) Model 3100 Atomic Absorption Spectrophotometer equipped with a PE HGA-600 Graphite Furnace and PE AS-60 Furnace Autosampler was used for Cu and Fe determinations in urine. Deuterium background correction was employed. A Cu or Fe hollow-cathode lamp (Perkin Elmer Corporation) was used and operated at either 10 W (Cu) or 15 W (Fe). The 324.8 nm atomic line was used for Cu and the 248.3 nm atomic line for Fe. The slit width for both Cu and Fe was 0.7 nm. Pyrolytically coated graphite tubes were used for all analyses. The injection volume was 20 µL. A typical graphite furnace temperature program is shown below.

GF-AAS temperature program

Procedure	*Temp / °C*	*Ramp / s*	*Hold / s*	*Int. Flow / mL min^{-1}*
Drying	90	1	5	300
	120	60	5	300
Pre-treatment	1250*	20	10	300
	20	1	10	300
Atomization – Cu / Fe	2300 / 2500	1	8	0
Post-treatment	2600	1	5	300

* A pre-treatment temperature of 1050 °C was used for tissue digest analyses

Cu, Fe and Zn in tissue digests were also determined at Hill Laboratories (Hamilton, New Zealand) using either a PE Sciex Elan-6000 or PE Sciex Elan-6100 DRC ICP-MS. The operating parameters are summarized in the table below.

Instrumental operating parameters for ICP-MS

Parameter	*Value*
Inductively coupled plasma	
Radiofrequency power	1500 W
Argon plasma gas flow rate	15 $l.min^{-1}$
Argon auxiliary gas flow rate	1.2 $l.min^{-1}$
Argon nebuliser gas flow rate	0.89 $l.min^{-1}$
Interface	
Sampler cone and orifice diameter	Ni / 1.1 mm
Skimmer cone and orifice diameter	Ni / 0.9 mm
Data acquisition parameters	
Scanning mode	Peak hopping
Dwell time	30 ms (Cu, Zn) / 100 ms (Fe)
Sweeps / replicate	20
Replicates	3
Sample uptake rate	1 $ml.min^{-1}$

Reagents: All reagents used were of the highest purity available and at least of analytical grade. GF-AAS standard working solutions of Cu and Fe were prepared by stepwise dilution of 1000 $mg.l^{-1}$ (Spectrosol standard solutions; BDH). Water was purified by a Millipore Milli-Q ultra-pure water system to a resistivity of 18 MΩ. Standard Reference Material 1577b Bovine Liver was obtained from the National Institute of Standards and Technology and used to evaluate the efficiency of tissue digestion. The results obtained are reported below.

GF-AAS and ICP-MS results for NIST SRM 1577b bovine liver*

Element	*Certified value*	*GF-AAS*	*ICP-MS*
Cu	160 ± 8	142 ± 12	164 ± 12
Fe	184 ± 15	182 ± 21	166 ± 14
Zn	127 ± 16	–	155 ± 42

* Measured in $\mu g.g^{-1}$ of dry matter.

Samples were pretreated as follows:

Urine: Urine was collected in pre-weighed 1.5 ml micro test tubes (eppendorf). After reweighing, the urine specimens were centrifuged and the supernatant diluted 25:1 with 0.02 M 69 % Aristar grade HNO_3. The sample was stored at 4 °C prior to GF-AAS analysis. If it was necessary to store a sample for a period in excess of 2 weeks, it was frozen and kept at –20 °C.

Heart: Following removal from the animal, the heart was cleaned of excess tissue, rinsed in buffer to remove excess blood, blotted dry and a wet ventricular weight recorded. Using titanium instruments a segment of left ventricular muscle was dissected and placed in a pre-weighed 5.0 ml polystyrene tube. The sample was freeze-dried overnight to constant weight before 0.45 ml of 69% Aristar grade HNO_3 was added. The sample tube was heated in a water bath at 65 °C for 60 minutes. The sample was brought to 4.5 ml with Milli-Q H_2O. The resulting solution was diluted 2:1 in order to reduce the HNO_3 concentration below the maximum permitted for ICP-MS analysis.

Serum: Terminal blood samples were centrifuged and serum treated and stored as per urine until analysis. From the trace metal content of serum from the terminal blood sample and urine collected over the final hour of the experiment, renal clearance was calculated using the following equation: renal clearance of trace metal = (a) the concentration of metal in urine ($\mu g.\ \mu l^{-1}$) times (b) the rate of urine flow ($\mu l.min^{-1}$), divided by (c) the concentration of metal in serum ($\mu g.\ \mu l^{-1}$)

Statistical analyses were as follows: All values are expressed as mean ± SEM and P values < 0.05 were considered statistically significant. Student's unpaired t-test was initially used to test for weight and glucose differences between the diabetic and control groups. For comparison of responses during drug exposure, statistical analyses were performed using analysis

of variance (Statistica for Windows v.6.1, SAS Institute Inc., Calfornia, USA). Subsequent statistical analysis was performed using a mixed model repeated measures ANOVA design.

Statistical analysis using a mixed linear model: Data for each dose level were analyzed using a mixed linear model (PROC MIXED; SAS, Version 8). The model included *diabetes*, *drug* and their interaction as fixed effects, *time* as a repeated measure, and *rats* as the subjects in the dataset. Complete independence is assumed across subjects. The full model was fitted to each dataset using a maximum likelihood estimation method (REML) fits mixed linear models (i.e., fixed and random effects models). A mixed model is a generalization of the standard linear model, the generalization being that you can analyse data generated from several sources of variation instead of just one. A level of significance of 0.05 was used for all tests.

The results were as follows.

Effects of STZ on blood glucose and body weight (Table 1): Blood glucose increased to 25 ± 2 $mmol.l^{-1}$ three days following STZ injection. Despite a greater daily food intake, diabetic animals lost weight whilst nondiabetic animals continued to gain weight during the 44 days following STZ/saline injection. On the day of the experiment blood glucose levels were 24 ± 1 and 5 ± 0 $mmol.l^{-1}$ and body weight 264 ± 7 g and 434 ± 9 g for diabetic and nondiabetic animals respectively.

Table 1. **Blood glucose, body weight and food consumption in diabetic versus nondiabetic animals.**

Diabetic animals n = 14, nondiabetic animals n = 14. Values shown as mean ± SEM. Asterisk indicates a significant difference ($P < 0.05$).

	Diabetic	Nondiabetic
Body weight prior to STZ/saline	303 ± 3 g	303 ± 3 g
Blood glucose 3 days following STZ/saline	*25 ± 2 mmol.l^{-1}	5 ± 0.2 mmol.l^{-1}
Daily food consumption	*58 ± 1 g	28 ± 1 g
Blood glucose on experimental day	*24 ± 1 mmol.l^{-1}	5 ± 0.2 mmol.l^{-1}
Body weight on experimental day	*264 ± 7 g	434 ± 9 g

Cardiovascular variables during infusion: Baseline levels of MAP during the control period prior to infusion were not significantly different between nondiabetic and diabetic animals (99 ± 4 mm Hg). HR was significantly lower in diabetic than nondiabetic animals (287 ± 11 and 364 ± 9 bpm respectively, $P < 0.001$). Infusion of trientine or saline had no effect on these variables except at the highest dose where MAP decreased by a maximum of 19 ± 4 mm Hg for the 2 min following administration and returned to pre-dose levels within 10 min. Body temperature and oxygen saturation remained stable in all animals throughout the experiment.

Urine excretion: Diabetic animals consistently excreted significantly more urine than nondiabetic animals except in response to the highest dose of drug (100 mg.kg^{-1}) or equivalent volume of saline (Fig. 16). Administration of the 100 mg.kg^{-1} dose of trientine also increased urine excretion in nondiabetic animals to greater than that of nondiabetic animals receiving the equivalent volume of saline (Fig. 17). This effect was not seen in diabetic animals.

Urinary excretion of Cu and Fe: Analysis of the dose response curves shows that, at all doses, diabetic and nondiabetic animals receiving drug excreted more Cu than animals receiving an equivalent volume of saline (Fig. 18). To provide

some correction for the effects of lesser total body growth of the diabetic animals, and thus to allow more appropriate comparison between diabetic and nondiabetic animals, excretion rates of trace elements were also calculated per gram of body weight. Figure 19 shows that diabetic animals had significantly greater copper excretion per gram of body weight in response to each dose of drug than did nondiabetic animals. The same pattern was seen in response to saline, however the effect was not always significant. Total copper excreted over the entire duration of the experiment was significantly increased in both nondiabetic and diabetic animals administered trientine compared with their respective saline controls (Fig. 20). Diabetic animals receiving drug also excreted more total copper per gram of body weight than nondiabetic animals receiving drug. The same significant trend was seen in response to saline administration (Fig. 21).

In comparison, iron excretion in both diabetic and nondiabetic animals receiving trientine was not greater than animals receiving an equivalent volume of saline (Fig. 22). Analysis per gram of body weight shows diabetic animals receiving saline excrete significantly more iron than nondiabetic animals, however this trend was not evident between diabetic and nondiabetic animals receiving trientine (Fig. 23). Total iron excretion in both diabetic and nondiabetic animals receiving drug was not different from animals receiving saline (Fig 24). In agreement with analysis of dose response curves, total iron excretion per gram of body weight was significantly greater in diabetic animals receiving saline than nondiabetic animals but this difference was not seen in response to trientine (Fig. 25).

Electron paramagnetic resonance spectroscopy showed that the urinary Cu from drug-treated animals was mainly complexed as trientine-Cu^{II} (Fig. 28), indicating that the increased tissue Cu

in diabetic rats is mainly divalent. These data indicate that rats with severe hyperglycaemia develop increased systemic Cu^{II} that can be extracted by selective chelation.

Serum content and renal clearance of Cu and Fe (Table 2): While there was no significant difference in serum copper content, there was a significant increase in renal clearance of copper in diabetic animals receiving drug compared with diabetic animals receiving saline. The same pattern was seen in nondiabetic animals, although the trend was not statistically significant (P = 0.056). There was no effect of drug or state (diabetic versus nondiabetic) on serum content or renal clearance of iron.

Table 2. Serum content and renal clearance of Cu and Fe in diabetic and nondiabetic animals receiving drug or saline.

	1.1.a.a.1 diabetic	*1.1.a.a.1 diabetic*	*1.1.a.a.2 nondiabetic*	*1.1.a.a.2 nondiabetic*
	trientine $n = 6$	*Saline* $n = 7$	*trientine* $n = 4$	*Saline* $n = 7$
Serum Cu ($\mu g.\mu l^{-1} \times 10^{-4}$)	7.56 ± 0.06	9.07 ± 1.74	7.11 ± 0.41	7.56 ± 0.62
Serum Fe ($\mu g.\mu l^{-1} \times 10^{-4}$)	35.7 ± 7.98	63.2 ± 16.4	33.6 ± 1.62	31.4 ± 8.17
Renal clearance Cu ($\mu l.min^{-1}$)	*28.5 ± 4.8	1.66 ± 0.82	19.9 ± 6.4	0.58 ± 0.28
Renal clearance Fe ($\mu l.min^{-1}$)	0.25 ± 0.07	0.38 ± 0.15	0.46 ± 0.22	0.11 ± 0.03

Values shown as mean ± SEM. Asterisk indicates a significant difference ($P < 0.05$) between diabetic animals receiving trientine and diabetic animals receiving an equivalent volume of saline.

Metal content of cardiac tissue (Table 3): Wet heart weights in diabetic animals were significantly less than those in nondiabetic animals while heart/body

weight ratios were increased. In some animals cardiac tissue was also analyzed for Cu and Fe content. There was no significant difference in content of copper between diabetic and nondiabetic animals receiving saline or trientine. Iron content of the non-diabetic animals administered saline was significantly greater than that of the diabetic animals administered saline.

Table 3. **Heart weight, heart weight/body weight ratios and trace metal content of heart tissue in diabetic versus nondiabetic animals.**

	Diabetic	Nondiabetic
Wet heart weight	*0.78 ± 0.02 g	1.00 ± 0.02 g
Heart weight/body weight	*2.93 ± 0.05 $mg.g^{-1}$	2.30 ± 0.03 $mg.g^{-1}$
Cu content $\mu g.g^{-1}$ dry tissue		
Trientine treated	24.7 ± 1.5	27.1± 1.0
Saline treated	21.3 ± 0.9	27.2 ± 0.7
Fe content $\mu g.g^{-1}$ dry tissue		
Trientine treated	186 ± 46	235 ± 39
Saline treated	†180 ± 35	274 ± 30

Diabetic animals: n = 5; nondiabetic animals: n = 10. Values shown as mean ± SEM. Asterisk indicates a significant difference ($P < 0.05$) between diabetic and non-diabetic animals.† indicates a significant difference ($P < 0.05$) between diabetic and non-diabetic animals receiving saline.

Results from application of a mixed linear model to the experimental analysis (Figure 27).

Copper: Diabetic rats excreted significantly higher levels of copper across all dose levels. Baseline copper excretion was also significantly higher in diabetic rats compared to and prior to drug administration. The drug resulted in a significantly higher excretion of copper compared to saline at all dose levels. There was no difference at baseline levels between the drug and saline groups. The interaction effect for the model was significant at dose levels of 1.0 $mg.kg^{-1}$ and above. The presence of a significant interaction term means that the influence of one

effect varies with the level of the other effect. Therefore, the outcome of a significant interaction between the diabetes and drug factors is increased copper excretion above the predicted additive effects of these two factors.

Iron: Diabetic rats in the saline only group excreted significantly higher levels of iron at all dose levels. This resulted in all factors in the model being significant across all dose levels.

In sum, the acute effect of intravenous trientine administration on the cardiovascular system and urinary excretion of copper and iron was studied in anesthetized, diabetic (6 weeks of diabetes, Streptozotocin induced) and nondiabetic rats. Animals were assigned to one of four groups: diabetic + trientine, diabetic + saline, nondiabetic + trientine, nondiabetic + saline. Drug, or an equivalent volume of saline, was administered hourly in doses of increasing strength (0.1, 1.0, 10, 100 $mg.kg^{-1}$) and urine was collected throughout the experiment in 15 min aliquots. A terminal blood sample was taken and cardiac tissue harvested. Analysis of urine samples showed the following main points:

- At all drug doses, diabetic and nondiabetic animals receiving drug excreted more Cu (μg) than animals receiving an equivalent volume of saline.
- When analyzed per gram of bodyweight, diabetic animals excreted significantly more copper ($\mu g.gBW^{-1}$) at each dose of trientine than did nondiabetic animals. The same pattern was seen in response to saline but the effect was not significant at every dose.
- At most doses, in diabetic animals iron excretion (μg) was greater in animals administered saline than in those administered drug. In nondiabetic animals there was no difference between iron excretion in response to saline or trientine administration.

- Analysis per gram of body weight shows no difference between iron excretion in nondiabetic and diabetic animals receiving trientine. Diabetic animals receiving saline excrete more iron per gram of bodyweight than nondiabetic animals receiving saline.
- Analysis of heart tissue showed no significant difference in total copper content between diabetic and nondiabetic animals, nor any effect of drug on cardiac content of iron and copper.
- Renal clearance calculations showed a significant increase in clearance of copper in diabetic animals receiving trientine compared with diabetic animals receiving saline. The same trend was seen in nondiabetic animals but the affect was not significant. There was no affect of trientine on renal clearance of iron.

Thus, there were no adverse cardiovascular effects were observed after acute administration of trientine. Trientine treatment effectively increases copper excretion in both diabetic and nondiabetic animals. The excretion of copper in urine following trientine administration is greater per gram of bodyweight in diabetic than in nondiabetic animals. Iron excretion was not increased by trientine treatment in either diabetic or nondiabetic animals.

Experiments relating to the efficacy of trientine to restore cardiac function in STZ diabetic rats were also carried out. As noted above, histological evidence from earlier studies showed that treatment with trientine appears to protect the hearts of diabetic Wistar rats from development of cardiac damage (diabetic cardiomyopathy), as judged by histology. However, it was unknown whether this histological improvement translates into an improvement in cardiac

function. One aim of this study was to use an isolated-working- rodent heart model to compare cardiac function in trientine-treated and non-treated, STZ diabetic and normal rats.

Male albino Wistar rats weighing 330-430g were assigned to four experimental groups as follows:

Experimental groups

Group	Code	N	Treatment
Group A	STZ	8	Diabetes for 13 weeks
Group B	STZ/D6	8	Diabetes for 13 weeks (Drug therapy week 7-13)
Group C	Sham	9	Non-diabetic controls
Group D	Sham/D7	11	Non-diabetic controls (Drug therapy week 7-13)

STZ = Streptozotocin; D7 = trientine treatment for 7 consecutive weeks commencing 6 weeks after the start of the experiment.

Diabetes was induced by intravenous streptozotocin (STZ; Sigma; St. Louis, MO). All rats were given a short inhalational anaesthetic (Induction: 5% halothane and 2L/min oxygen, maintained on 2% halothane and 2 L/min oxygen). Those in the two diabetic groups then received a single intravenous bolus dose of STZ (57mg/kg body weight) in 0.5 ml of 0.9% saline administered via a tail vein. Non-diabetic sham-treated animals received an equivalent volume of 0.9% saline. Diabetic and non-diabetic rats were housed in like-pairs and provided with free access to normal rat chow (Diet 86 pellets; New Zealand Stock Feeds, Auckland, NZ) and deionized water *ad libitum*. Each cage had two water bottles on it to ensure equal access to water or drug for each animal. Animals were housed at 21 degrees and 60% humidity in standard rat cages with a sawdust floor that was changed daily.

Blood glucose was measured in tail-tip capillary blood samples (Advantage II, Roche Diagnostics, NZ Ltd). Sampling was performed on all groups at the same time of the day. Blood glucose and body weight were measured on day 3 following STZ/saline injection and then

weekly throughout the study. Diabetes was confirmed by presence of polydipsia, polyuria and hyperglycemia (>11mmol.L^{-1}).

In the drug treated diabetic group, trientine was prepared in the drinking water for each cage at a concentration of 50mg/L. Each animal consumed about 260ml water per day once diabetes was established, to yield a total drug dose per animal per day of ~13mg/kg. The trientine-containing drinking water was administered continuously from the start of week 7 until the animal was sacrificed at the end of week 13. In the case of the Sham/D7 non-diabetic group that drank less water per day than diabetic animals, the drug concentration in their drinking water was adjusted each week so that they consumed approximately the same dose as the corresponding STZ/D7 group. At the time the drug started in the diabetic group the diabetic animals were expected to have to have established cardiomyopathy, as shown by preliminary studies (data not shown) and confirmed in the literature. See Rodrigues B, *et al.*, *Diabetes* 37(10):1358-64 (1988).

On the last day of the experiment, animals were anesthetized (5% halothane and 2L.min^{-1} O_2), and heparin (500 IU.kg^{-1}) (Weddel Pharmaceutical Ltd., London) administered intravenously via tail vein. A 2ml blood sample was then taken from the inferior vena cava and the heart was then rapidly excised and immersed in ice-cold Krebs-Henseleit bicarbonate buffer to arrest contractile activity. Hearts were then placed in the isolated perfused working heart apparatus.

The aortic root of the heart was immediately ligated to the aortic cannula of the perfusion apparatus. Retrograde (Langendorff) perfusion at a hydrostatic pressure of 100 cm H_2O and at 37^{o}C was established and continued for 5min while cannulation of the left atrium via the pulmonary vein was completed. The non-working (Langendorff) preparation was then converted to the working heart model by switching the supply of perfusate buffer from the aorta to the left atrium at a filling pressure of 10 cm H_2O. The left ventricle spontaneously ejected into the aortic cannula against a hydrostatic pressure (after-load) of 76 cmH_2O (55.9mmHg). The

perfusion solution was Krebs-Henseleit bicarbonate buffer (mM: KCl 4.7, $CaCl_2$ 2.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, NaCl 118, and $NaHCO_3$ 25), pH 7.4 containing 11mM glucose and it was continuously gassed with 95% O_2:5% CO_2. The buffer was also continuously filtered in-line (initial 8μm, following 0.4μm cellulose acetate filters; Sartorius, Germany). The temperature of the entire perfusion apparatus was maintained by water jackets and buffer temperature was continuously monitored and adjusted to maintain hearts at 37°C throughout perfusion.

A modified 24g plastic intravenous cannula (Becton Dickson, Utah, USA) was inserted into the left ventricle via the apex of the heart using the normal introducer-needle. This cannula was subsequently attached to a SP844 piezo-electric pressure transducer (AD Instruments) to continuously monitor left ventricular pressure. Aortic pressure was continuously monitored through a side arm of the aortic cannula with a pressure transducer (Statham Model P23XL, Gould Inc., CA, USA). The heart was paced (Digitimer Ltd, Heredfordshire, England) at a rate of 300bpm by means of electrodes attached to the aortic and pulmonary vein cannulae using supra-threshold voltages with pulses of 5-ms duration from the square wave generator.

Aortic flow was recorded by an in-line flow meter (Transonic T206, Ithaca, NY, USA) and coronary flow was measured by timed 30sec collection of the coronary vein effluent at each time point step of the protocol.

The working heart apparatus used was a variant of that originally described by Neely, JR, *et al.*, *Am J Physiol* **212**:804-14 (1967). The modified apparatus allowed measurements of cardiac function at different pre-load pressures (Figure 14 and Figure 15). This was achieved by constructing the apparatus so that the inflow height of the buffer coming to the heart could be altered through a series of graduated steps in a reproducible manner. As in the case of the pre-load, the outflow tubing from the aorta could also be increased in height to provide a series of defined after-load pressures. The after-load heights have been converted to mm Hg for presentation in the results which is in keeping with published convention.

All data from the pressure transducers and flow probe were collected (Powerlab 16s data acquisition machine; AD Instruments, Australia). The data processing functions of this device were used to calculate the first derivative of the two pressure waves (ventricular and aortic). The final cardiac function data available comprised:

Cardiac output*; aortic flow; coronary flow; peak left ventricular/aortic pressure developed; maximum rate of ventricular pressure development (+dP/dt)**; maximum rate of ventricular pressure relaxation (-dP/dt)**; maximum rate of aortic pressure development (aortic +dP/dt); maximum rate of aortic relaxation (aortic -dP/dt).

[*Cardiac output (CO) is the amount of buffer pumped per unit time by the heart and is comprised of buffer that is pumped out the aorta as well as the buffer pumped into the coronary vessels. This is an overall indicator of cardiac function. ** +dP/dt is the rate of change of ventricular (or aortic pressure) and correlates well with the strength of the contraction of the ventricle (contractility). It can be used to compare contractility abilities of different hearts when at the same pre-load (Textbook of Medical Physiology, Ed. A.Guyton. Saunders company 1986). -dP/dt is an accepted measurement of the rate of relaxation of the ventricle].

The experiment was divided into two parts, the first with fixed after-load and variable pre-load the second, which immediately followed on from the first, with fixed pre-load and variable after-load.

Fixed After-load and changing Pre-load: After the initial cannulation was completed, the heart was initially allowed to equilibrate for 6min at 10cm H_2O atrial filling pressure and 76cm H_2O after-load. During this period the left ventricular pressure transducer cannula was inserted and the pacing unit started. Once the heart was stable, the atrial filling pressure was then reduced to 5cm H_2O of water and then progressively increased in steps of 2.5cmH_2O over a series of 7 steps to a maximum of 20cmH_2O. The

pre-load was kept at each filling pressure for 2min, during which time the pressure trace could be observed to stabilize and the coronary flow was measured. On completion of the variable pre-load experiment, the variable after-load portion of the experiment was immediately commenced.

Fixed Pre-load and changing After-load: During this part of the experiment the filling pressure (pre-load) was set at 10cm H_2O and the after-load was then increased from 76cm H_2O (55.9 mm Hg) in steps of 8cm H_2O (5.88mmHg); again each step was of 2min duration. The maximum height (after-load) to which each individual heart was ultimately exposed, was determined either by attainment of the maximal available after-load height of 145cm H_2O (106.66 mm Hg), or the height at which measured aortic flow became 0 ml/min. In the later situation, the heart was considered to have "functionally failed." To ensure that this failure was indeed functional and not due to other causes (*e.g.*, permanent ischaemic or valvular damage) all hearts were then returned to the initial perfusion conditions (pre-load 10cm H2O; after-load 75 cm H2O) for 4 minutes to confirm that pump function could be restored. At the end of this period the hearts were arrested with a retrograde infusion of 0.4ml of cold KCL (24mM). The atria and vascular remnants were then excised, the heart blotted dry and weighed. The ventricles were incised midway between the apex and atrioventricular sulcus. Measurements of the ventricular wall thickness were then made using a micro-caliper (Absolute Digimatic, Mitutoyo Corp, Japan).

Data from the Powerlab was extracted by averaging 1min intervals from the stable part of the electronic trace generated from each step in the protocol. The results from each group were then combined and analyzed for differences between the groups for the various cardiac function parameters (aortic flow, cardiac flow, MLVDP, LV or aortic +/-dP/dt). Differences between repeated observations at different pre-load conditions were

explored and contrasted between study group using a mixed models approach to repeated measures (SAS v8.1, SAS Institute Inc, Cary NC). Missing random data were imputed using a maximum likelihood approach. Significant mean and interaction effects were further examined using the method of Tukey to maintain a pairwise 5% error rate for post hoc tests. All tests were two-tailed. Survival analysis was done using Proc Liftest (SAS V8.2). A one-way analysis of variance was used to test for difference between groups in various weight parameters. Tukey's tests were used to compare each group with each other. In each graph unless otherwise stated.* indicates $p<0.05$ = STZ v STZ/D7, #.$p<0.05$ = STZ/D7 v Sham/D7.

Results showing that the weights of the animals at the end of the experimental period are found in Table 4. Diabetic animals were about 50% smaller than their corresponding age matched normals. A graph of the percentage change in weight for each experimental group is found in Figure 5, wherein the arrow indicates the start of trientine treatment.

Table 4. Initial and final animal body weights (mean ± SD)

	Number (n)	Treatment	Initial weight (g)	Final weight (g)
Group A	9	STZ	361 ±12	221±27
* Group B	8	STZ/D7	401 ± 33	290±56
* Group C	8	Sham	361 ±16	574±50
Group F	11	Sham/D7	357 ±7	563±17

*P < 0.05

Blood glucose values for the three groups of rats are presented in Figure 6. Generally, the presence of diabetes was established and confirmed within 3-5 days following the STZ injection. The Sham and Sham/D7 control group remained normoglycemic throughout the experiment. Treatment with the drug made no difference to the blood glucose profile (p=ns) in either treated group compared to their respective appropriate untreated comparison group.

Final heart weight and ventricular wall thickness measurements are presented in Table 5. There was a small but significant improvement in the "heart : body weight" ratio with treatment in the diabetic animals. There was a trend toward improved "ventricular wall thickness:bodyweight" ratio in treated diabetics compared to non-treated but this did not reach significance.

Table 5 Final heart weights (g) and per g of animal body Weight (BW) (mean ± SD)

Group	Heart weight (g)	Heart weight (g) /BW (g)	Left Ventricular wall thickness (mm)	Left Ventricular wall thickness per BW (mm)/ (g)
Sham	1.58 ± 0.13§	0.0028±0.0002§	3.89±0.38§	0.0068±0.0009§
STZ/D7	1.18 ± 0.24	0.0041±0.0005	3.79±0.52	0.0127±0.0027
STZ	1.03 ± 0.17	0.0047±0.0004	3.31±0.39	0.0152±0.0026
Sham/D7	1.58 ± 0.05§	0.0028±0.0001§	4.03±0.1§	0.0072±0.0003§

* P<0.05

§ = significant with the STZ and STZ/D7 groups p<0.05

Part I results: The following graphs of Figures 7 to 12 represent cardiac performance parameters of the animals (STZ diabetic; STZ diabetic +drug; and sham-treated controls) while undergoing increasing atrial filling pressure (5-20 cmH_2O, pre-load) with a constant after-load of 75cm H_2O. All results are mean ± sem. In each graph for clarity unless otherwise stated, only significant differences related to the STZ/D7 the other groups are shown:* indicates p<0.05 for STZ v STZ/D7, # p<0.05 for STZ/D7 v Sham/D7. Unless stated, STZ/D7 v Sham or Sham/D7 was not significant.

Cardiac output (Figure 7) is the sum to the aortic flow (Figure 10) and the coronary flow as displayed in Figure 8. Since the control hearts and experimental groups have significantly different final weights, the coronary flow is also presented (Figure 9) as the flow

normalized to heart weight (note that coronary flow is generally proportional to cardiac muscle mass, and therefore to cardiac weight)

The first derivative of the pressure curve gives the rate of change in pressure development in the ventricle with each cardiac cycle and the maximum positive rate of change (+dP/dt) value is plotted in Figure 11. The corresponding maximum rate of relaxation (-dP/dt) is in Figure 12. Similar results showing improvement in cardiac function were found from the data derived from the aortic pressure cannula (results not shown).

Part II results:

Under conditions for constant pre-load and increasing after-load the ability of the hearts to cope with additional after-load work was assessed. The plot of functional survival, that is the remaining number of hearts at each after-load that still had an aortic output of greater than 0ml/min is found in Figure 13 and Table 6.

Table 6. Cardiac survival at each after-load pressure

Number surviving (aortic flow >0mls/min)					Percentage functioning at each afterload			
Afterload (mmHg)	STZ	STZ/D7	Sham	Sham/D 7	STZ	STZ/D7	Sham	Sham/D7
55.9	8	8	9	11	100%	100%	100%	100%
61.8	8	8	9	11	100%	100%	100%	100%
67.7	8	8	9	11	100%	100%	100%	100%
71.4	6	8	9	11	75%	100%	100%	100%
77.2	5	8	9	11	63%	100%	100%	100%
83.1	4	8	9	11	50%	100%	100%	100%
88.3	3	7	9	11	38%	88%	100%	100%
94.9	1	6	9	11	13%	75%	100%	100%
100.8	0	5	9	11	0%	63%	100%	100%
106.7	0	1	9	9	0%	13%	100%	82%

Cu chelation normalizes LV structure in diabetic rats

Following functional analysis, LV histology was studied by laser confocal (LCM; Fig. 29a - d) and transmission electron microscopy (TEM; Fig 29e - h). For LCM, LV sections were co-stained with phalloidin to visualize actin filaments, and β_1-integrin as a marker for the extracellular space. Ding B, et al., "Left ventricular hypertrophy in ascending aortic stenosis in mice: anoikis and the progression to early failure," *Circulation* **101**:2854-2862 (2000).

For each treatment, 5 sections from each of 3 hearts were examined by both LCM and TEM. For LCM, LV sections were fixed (4% paraformaldehyde, 24 h); embedded (6% agar); vibratomed (120 pm, Campden); stained for f-actin (Phalloidin-488, Molecular Probes) and β_1-integrin antibody with a secondary antibody of goat anti-rabbit conjugated to CY5 (1:200; Ding B, et al., "Left ventricular hypertrophy in ascending aortic stenosis in mice: anoikis and the progression to early failure," *Circulation* **101**:2854-2862 (2000)); and visualised (TCS-SP2, Leica). For TEM, specimens were post-fixed (1:1 v/v 1% w/v 0s0 M 0s0 M PBS); stained (aqueous uranyl acetate (2 % w/v, 20 mm) then lead citrate (3 mm)); sectioned (70 nm); and visualized (CM-12, Phillips).

Compared with controls (Fig. 29a), diabetes caused obvious alterations in myocardial structure, with marked loss of myocytes; thinning and disorganization of remaining myofibrils; decreased density of actin filaments; and marked expansion of the interstitial space (Fig. 29b). These findings are consistent with previous reports. Jackson CV, et al., "A functional and ultrastructural analysis of experimental diabetic rat myocardium: manifestation of acardiomyopathy," *Diabetes* **34**:876-883 (1985). By marked contrast, myocardial histology following trientine treatment was essentially normal (Fig. 29c). Importantly, the orientation and volume of card iomyocytes and their actin filaments was largely normalized, consistent with the normalization of $-dP_{LV}/dt$ observed in the functional studies. Trientine treatment reversed the expanded cardiac ECM. Myocardium from trientine-treated non-diabetics appeared normal by LCM (Fig. 29d) suggesting that it has no detectable adverse effects on LV structure. Thus, Cu chelation essentially restored the normal histological appearance of the myocardium without suppressing hyperglycaemia. These data provide important structural correlates for the functional recovery of these hearts, shown above.

TEM was largely consistent with LCM. Compared with controls (Fig. 29e), diabetes caused unmistakable myocardial damage characterized by loss of myocytes with evident myocytolysis; disorganization of remaining cardiomyocytes in which swollen mitochondria were prominent; and marked expansion of the extracellular space (Fig. 29f). These findings are

consistent with previous reports. Jackson CV, et al., "A functional and ultrastructural analysis of experimental diabetic rat myocardium: manifestation of acardiomyopathy," *Diabetes* 34:876-883 (1985). Oral trientine caused substantive recovery of LV structure in diabetics, with increased numbers and normalized orientation of myocytes; return to normal of mitochondrial structure; and marked narrowing of the extracellular space (Fig. 29g). These data suggest that hyperglycaemia-induced systemic Cu^{II} accumulation might contribute to the development of mitochondrial dysfunction. Brownlee M, "Biochemistry and molecular cell biology of diabetic complications," *Nature* 414:813-820 (2001). Myocardium from trientine-treated non- diabetics appeared normal by TEM (Fig. 29h). Thus, trientine treatment normalized both cellular and interstitial aspects of hyperglycaemia-induced myocardial damage. Taken together, these microscopic studies provide remarkable evidence that selective Cu- chelation can normalize LV structure, even in the presence of severe chronic hyperglycaemia.

IN SUM, FOR EXAMPLE,

- Treatment with trientine had no obvious effect on blood glucose concentrations in the two diabetic groups (as expected).
- There was a small but significant improvement in the (heart weight) / (body weight) ratio in the trientine-treated diabetic group compared to that of the untreated diabetic group.
- When the Pre-load was increased with the After-load held constant, cardiac output was restored to Sham values. Both the aortic and absolute coronary flows improved in the drug treated group.
- Indicators for ventricular contraction and relaxation were both significantly improved in the drug treated group compared to equivalent values in the untreated diabetic group. The improvement restored function to such an extent that there was no significant difference between the drug treated and the sham-treated control groups.
- The aortic transducer measures of pressure change also showed improved function in the drug treated diabetic group compared to the untreated diabetics (data not shown).

- When after-load was increased in the presence of constant pre-load, it was observed that the heart's ability to function at higher after-loads was greatly improved in the drug treated diabetic group compared to the untreated diabetic group. When 50% of the untreated diabetic hearts had failed, about 90% of the trientine treated diabetic hearts were still functioning.
- Compared to the untreated diabetic hearts, the response of the drug treated diabetic hearts showed significant improvements in several variables: cardiac output, aortic flow, coronary flow, as well as improved ventricular contraction and relaxation indices.
- Drug treatment of normal animals had no adverse effects on cardiac performance.

It is concluded that treatment of STZ diabetic rats with trientine dramatically improves several measures of cardiac function. It is also concluded that administration of oral trientine for 7 weeks in Wistar rats with previously established diabetes of 6 weeks duration resulted in a global improvement in cardiac function. This improvement was demonstrated by improved contractile function (; +dP/dT) and a reduction in ventricular stiffness (-dP/dT). The overall ability of the Trientine treated diabetic heart to tolerate increasing after-load was also substantially improved.

- **HUMAN STUDIES – Phase II**

Trace element balance in humans

Human studies were approved by institutional ethics and regulatory committees. Males with uncomplicated T2DM (Table 9) underwent 12-d elemental balance studies in a fully-residential metabolic unit. All foods and beverages were provided.

Total daily intake (method of double diets) and excretion (urinary and faecal) of trace elements (Ca, Mg, Zn, Fe, Cu, Mn, Mo, Cr and Se) were determined (ICP MS). Baseline measurements were taken during the first 6 d, after which oral trientine (2.4 g once-daily) or

matched placebo was administered in a 2 x 2 randomised double-blind protocol and metal losses measured for a further 6 d.

Effects of chronic trientine treatment on LV mass in humans

Subjects (30-70 y) who provided written informed consent were eligible for inclusion if they had:

T2DM with HbA_{1c} >7%; cardiac ejection fraction (echocardiography) =45% with evidence of diastolic dysfunction but no regional wall-motion anomalies; no new medications for more than 6 months with no change of ß-blocker dose; normal electrocardiogram (sinus rhythm, normal PR Interval, normal T wave and QRS configuration, and isoelectric ST segment); and greater than 90% compliance with single-blinded placebo therapy during a 2-w run-in period). Women were required to be post-menopausal, surgically sterile, or non-lactating and non-pregnant and using adequate contraception. Patients were ineligible if they failed to meet the inclusion criteria or had: morbid obesity (B. M. I. = 45 $kg.m^{-2}$ T1 DM; a history of significant cardiac valvular disease; evidence of autonomic neuropathy; ventricular wall motion abnormality; history of multiple drug allergies; use or misuse of substances of abuse; abnormal laboratory tests at randomisation; or standard contraindications to MRI.

Before randomisation, potentially eligible subjects entered a 4-w single blind run-in phase of two placebo-capsules twice-daily and underwent screening echocardiography, being excluded if regional wall motion abnormalities or impaired LV systolic function (ejection fraction <50%) were detected. In addition, LV diastolic filling was assessed using mitral inflow Doppler (with pre-load reduction) to ensure patients had abnormalities of diastolic filling; no patient with normal mitral filling proceeded to randomisation. Subjects meeting inclusion criteria and with no grounds for exclusion were then randomised to receive trientine (600 mg twice-daily) before meals (total dose 1.2 $g.d^{-1}$) or 2 identical placebo capsules twice-daily before meals, in a double-blind, parallel-group design. Treatment assignment was performed centrally using variable block sizes to

ensure balance throughout trial recruitment and numbered drug packs were prepared and dispensed sequentially to randomised patients. The double-blind treatment was continued for 6 months in each subject.

At baseline and following 6 months' treatment, LV mass was determined using cardiac MRI, performed in the supine position with the same 1.5 T scanner (Siemens Vision) using a phased array surface coil. Prospectively gated cardiac cine images were acquired in 6 short axis and 3 long axis slices with the use of a segmented k-space pulse sequence (TR 8 ms; TE 5 ms; flip angle 10°; field of view 280 - 350 mm) with view sharing (11 - 19 frames.slice^{-1}). Each slice was obtained during a breath-hold of 15 - 19 heart beats. The short axis slices spanned the left ventricle from apex to base with a slice thickness of 8 mm and inter-slice gap of 2 - 6 mm. The long axis slices were positioned at equal 60° intervals about the long axis of the LV. Cardiac MRI provides accurate and reproducible estimates of LV mass and volume. LV-mass and volume were calculated using guide point modelling, which produces precise and accurate estimations of mass and volume. Briefly, a three dimensional mathematical model of the LV was interactively fitted to the epicardial and endocardial boundaries of the LV wall in each slice of the study, simultaneously. Volume and mass were then calculated from the model by numerical integration (mass = wall volume x 1.05 g.ml^{-1}).. All measurements were performed by 1 measurer at the end of six months' data collection. Outcome analyses were conducted by intention-to-treat, using a maximum likelihood approach to impute missing at random data within a mixed model, and marginal least-squares adjusted-means were determined. Changes from baseline were compared between treatment-groups in the mixed model with baseline values entered as covariate. Since there were only 2 groups in the main effect and no interaction effect, no *post hoc* procedures were employed. In additional analysis the influence of clinically important differences between the treatment groups at baseline was considered by adjusting for them as covariates in an additional model. All *P* values were calculated from 2-tailed tests of statistical significance and a 5%

significance level was maintained throughout. The effect of treatment on categorical variables was tested using the procedures of Mantel and Haenzel (SAS v8.01, SAS Institute).

Table 7 shows baseline information on 30 patients with long-standing type 2 diabetes, no clinical evidence of coronary artery disease and abnormal diastolic function who participated in a 6-month randomised, double blind, placebo controlled study of chronic oral therapy with trientine dihydrochloride.

- **Table 7: Characteristics of Study Participants**

	Placebo	Trientine dihydrochloride
N	15	15
Median age (years)	54 (range 43-64)	52 (range 33-69)
% female	44%	56%
Median duration of diabetes (years)	10 (6-24)	8 (4-15)
Mean body mass index (kg/m^2)	32 (SD 5)	34 (SD 5)
% hypertensive	64%	80%
% HbA_{1c}>8	93%	80%

MRI scans of the heart at baseline and 6-months showed a significant reduction in LV mass and a significant improvement in diastolic function measured as a change in apical rotation (AR) at the end of systole. See, Table 8. These effects indicate improved structure and function in the human heart following 6 months of trientine therapy.

Table 8 : Phase II: INFO-Cardiac

MRI Results

	Placebo (n=15)	GC811007 (n=15)	P
Baseline LVM	202.17	207.45	0.778
Δ LVM 1-6mo	+6.57	-10.49	0.0045

Baseline AR	12.37	12.49	0.931
Δ AR 1-6mo	+0.81	-2.19	0.029

Therefore, an equivalent dose of oral trientine dihydrochloride corrected for weight (15 mg/kg) is effective in both rats and humans.

With reference to Figures 30 and 31 there is shown the plasma concentration – time profiles of trientine after oral administration. The plasma concentration was determined using the process as defined in Miyazaki K, et al., "Determination of trientine in plasma of patients with high-performance liquid chromatography," *Chem. Pharm. Bull.* 38:1035-1038 (1990).

Urinary Cu losses are increased following oral trientine treatment in humans with type-2 diabetes

We measured urinary metal excretion in human males with T2DM or matched non-diabetic controls, baseline information on which is shown in Table 9, in a randomised, double blind, placebo-controlled trial.

- **Table 9: Characteristics of Study Participants**

	Placebo control	Trientine treated control	Placebo diabetic	Trientine treated diabetic
Median age (years)	42 (range 32 – 53)	52 (range 30 – 68)	51 (range 32 – 66)	50 (range 30 - 64)
n	10	10	10	10
Median duration of diabetes (years)	-	-	5.9 (range 1 - 13)	7.5 (range 1 - 34)
Fasting blood glucose ($mmol.L^{-1}$)	4.7 ± 0.3	5.0 ± 0.3	11.5 ± 3.8	10.8 ± 4.4
Mean HbA_{1c} (%)	5.4 ± 0.3	5.0 ± 0.3	9.9 ± 2.8	9.1 ± 1.6
Body mass index ($kg.m^{-2}$)	24.6 ± 3.5	27.9 ± 5.2	32.8 ± 4.4	30.4 ± 3.2

(mean ± S. E. M. unless otherwise stated); f. p. g., HbA_{1c} and B. M. I. were significantly greater in diabetics and groups were otherwise well-matched).

Basal 2-h Cu-losses were measured for 10 h in diabetic (n = 20) and matched control (n = 20) subjects during part of day I (Fig. 32); and daily losses were determined throughout days 1 - 6. Urine volumes were equivalent in drug- and placebo-treated groups. Baseline urinary Cu-excretion was significantly greater in diabetics than controls (mean diabetic, 0.257 $\mu mol.d^{-1}$ control, 0.196; $P < 0.001$). Trientine- and placebo-evoked 2-h urinary Cu-excretion was measured again in the same subjects on day 7 following oral drug (2.4 g once-daily) or matched placebo (n = $10.group^{-1}$. Trientine increased urinary Cu in both groups, but the excretion rate in diabetes was greater (Fig 30; $P < 0.05$). There was no corresponding increase in trientine-evoked urinary Fe excretion, although basal concentrations in diabetes were increased relative to control ($P < 0.001$; results not shown). Thus, trientine elicited similar urinary Cu responses in rats with T1DM and in humans with T2DM. Mean trientine-evoked urinary Cu-excretion was 5.8 $\mu mol.d^{-1}$; in T2DM compared to 4.1 in non-diabetic controls, a 40 % increase. This correspondence between the two major forms of diabetes in two species suggests that increased systemic Cu^{II} is likely to be widely present in diabetes.

Trientine treatment reverses LVH in type-2 diabetic humans

We determined the effect of chronic trientine on LV mass in adults with T2DM, baseline information of which is shown in Table 10.

Table 10 Characteristics of Study Participants

	Placebo	Trientine-treated
Median age (years)	54 (range 43 – 64)	52 (range 33 - 69)
N	15	15
Female (%)	44	56
Median duration of diabetes (years)	10 (range 1- 24)	8 (range 1 -21)

Mean % HbA_{1c} (SD)	9.3 (1.3)	9.3 (2.0)
Initial left ventricular mass (g) (SD)	202.2 (53.1)	207.5 (48.7)
Δ urinary copper ($\mu mol.L^{-1}$)	0.67 (-1.16 to 2.49)	-0.83 (-2.4 to 0.74)
Δ systolic blood pressure (mmHg)	-1.9 (-10.6 to 6.8)	-3.5 (-9.5 to 1.8)
Δ diastolic blood pressure (mmHg)	-4.5 (-9.0 to 0.01)	-3.9 (-13.4 to 6.5)
Δ left ventricular mass/body surface area ($g.m^{-2}$)	+3.49 (0.63 to 7.61)	-5.56** (-9.64 to -1.48)

baseline values (*above line*); differences in key treatment-variables (6 months — baseline, mean (95% confidence interval; *below line*: **, $P < 0.01$ vs. placebo).

Trientine (600 mg twice-daily, a dose at the lower end of those employed in adult Wilson's disease, see Dahlman T, et al., "Long-term treatment of Wilson's disease with triethylene tetramine dihydrochloride (trientine)," *Quart. J. Med* **88**: 609-616 (1995)) or placebo were administered orally for 6 months to equivalent groups of diabetic adults ($n = 15.group^{-1}$; Table 10), also matched for pharmacotherapy including: β-blockers, calcium antagonists, ACE-inhibitors, cholesterol-lowering drugs, antiplatelet agents and antidiabetic drugs. LV masses were determined by tagged-molecular resonance imaging (MRI; see Bottini PB, et al., "Magnetic resonance imaging compared to echocardiography to assess left ventricular mass in the hypertensive patient," *Am. J. Hypertens* **8**: 221-228 (1995)) at baseline and following 6 months' trientine treatment. As expected, diabetics initially had significant LVH, consistent with previous reports. Struthers AD & Morris AD, "Screening for and treating left-ventricular abnormalities in diabetes mellitus: a new way of reducing cardiac deaths," *Lancet* **359**: 1430-1432 (2002). Mean LV mass significantly decreased, by 5%, following 6 months' trientine treatment, whereas that in placebo-treated subjects increased by 3% (Fig. 33); this highly significant effect remained after LV mass was indexed to body surface area, and occurred without change in systolic or diastolic

blood pressure (Table 10). Thus, trientine caused powerful regression in LV mass without altering blood pressure or urinary volume (Fig. 32). No significant drug-related adverse events occurred during the 6 months' trientine therapy.

These data, taken together with those in rats above, indicate that chronic Cu chelation can cause significant regeneration of the heart in those with diabetes-evoked damage.

It is concluded that rats and humans with diabetes acquire increased systemic Cu^{II}, which can be removed by treatment with the Cu-selective chelator, trientine. Trientine largely reversed heart failure and LV damage in severely diabetic rats. Furthermore, 6 months' oral trientine therapy significantly ameliorated left ventricular hypertrophy in humans with type-2 diabetes.

It will be understood, however, that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the host undergoing therapy.

The compounds of the present invention can be combined with other compounds having related utilities to prevent and treat tissue damage or excess tissue copper.

***

All patents, publications, scientific articles, web sites, and other documents and materials referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such referenced document and material is hereby incorporated by reference to the same extent as if it had been incorporated by reference in its entirety individually or set forth herein in its entirety. Applicants reserve the right to physically incorporate into this specification any and all materials and information from any such patents,

publications, scientific articles, web sites, electronically available information, and other referenced materials or documents.

The specific methods and compositions described herein are representative of preferred embodiments and are exemplary and not intended as limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. Thus, for example, in each instance herein, in embodiments or examples of the present invention, any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms in the specification. As used herein the term "and/or" means both "and" and "or". As used herein the addition of "(s)" as part of a word embraced both the singular and plural of that word. Also, the terms "comprising", "including", containing", *etc.* are to be read expansively and without limitation. The methods and processes illustratively described herein suitably may be practiced in differing orders of steps, and that they are not necessarily restricted to the orders of steps indicated herein or in the claims. It is also that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Under no circumstances may the patent be interpreted to be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

DOSING REGIMEN FOR TRIENTINE

The half life of trientine, indicated for the treatment and reversal of heart failure and coronary heart disease, is relatively short – being approximately 2 hours. To maintain optimal blood levels, either multiple dose regimen, or a controlled release preparation requiring fewer doses per day is required.

With reference to Figures 36 and 37 there is shown the plasma concentration – time profiles of trientine after oral administration. The plasma concentration was determined using the process as defined in Miyazaki, K., et al., Determination of trientine in plasma

of patients with high-performance liquid chromatography. Chem Pharm Bull, 1990. 38:p. 1035-38.

Ideally trientine should be taken in addition to current therapies, at a maximum tolerated dose, utilizing a dose regimen which fits its pharmacokinetic profile. Patients with heart failure and/or coronary artery disease are frequently on multiple drug regimens. Therefore, a controlled release preparation requiring fewer doses per day is preferred. The proof of principle Phase 2 study has shown positive results. However, the dosage regimen was sub-optimal when compared with its pharmacokinetic profile and the study does not assure the efficacy of the drug which would be required in pivotal trials by regulatory authorities.

For these purposes, the crystalline trientine dihydrochloride salt may be administered parenterally (including subcutaneous injections, intravenous, intramuscular, intradermal injection or infusion techniques) or by inhalation spray in dosage unit formulations containing conventional non-toxic pharmaceutically-acceptable carriers, adjuvants and vehicles.

Routes for parenteral administration therefore include intravenous, intramuscular, intraperitoneal, subdermal, and subcutaneous administration.

The injectable solutions or suspensions may be formulated according to known art, using suitable non-toxic, parenterally-acceptable diluents or solvents, such as mannitol, 1,3-butanediol, water, Ringer's solution or isotonic sodium chloride solution, and/or suitable dispersing or wetting and suspending agents, such as sterile, bland, fixed oils, including synthetic mono-or diglycerides, and fatty acids, including oleic acid.

Suitable dispersing or suspending agents for aqueous suspensions include synthetic and natural gums such as tragacanth, acacia, alginate, dextran, sodium carboxymethylcellulose, methylcellulose, polyvinyl-pyrrolidone or gelatin.

A formulation for injection, infusions, etc. may include such preservatives and other inclusions desired having regard to shelf life requirements and mode of administration.

The pharmaceutical preparation may also contain non-toxic auxillary substances such as antibacterial components for example quaternary ammonium compounds, known

to have cold sterilizing properties and which are non-injurious in use, such as thimerosal, methyl and propyl paraben, benzyl alcohol, phenyl ethanol.

Other suitable additions to the injectable solution include antibacterial preservatives, buffers, solubilizers, antioxidants, and other pharmaceutical adjuncts.

Extended-release formulations containing trientine or salts thereof suitable for parenteral administration. Extended rates of drug action following injection may be achieved in a number of ways, including the following: crystal or amorphous drug forms having prolonged dissolution characteristics; slowly dissolving chemical complexes of the drug entity; solutions or suspensions of drug in slowly absorbed carriers or vehicles (as oleaginous); increased particle size of drug in suspension; or, by injection of slowly eroding microspheres of drug (for example, see: Friess, W., Lee, G. and Groves, M. J. Insoluble collagen matrices for prolonged delivery of proteins. Pharmaceut Dev Technol 1996;1:185-193). The duration of action of the various forms of insulin for example is based in part on its physical form (amorphous or crystalline), complex formation with added agents, and its dosage form (solution of suspension).

In addition to the above means of achieving extended drug action, the rate and duration of drug delivery may be controlled by slow intravenous or subcutaneous infusion, using mechanically-controlled drug infusion pumps. Reference is made herein to our simultaneous filing on the slow release and/or other related means of administration as defined in that patent specification.

Further examples of parenteral products with long-acting properties are available from the USP (supra).

Yet further embodiments of the invention include the incorporation of trientine or its salts into suppositories (rectal or vaginal), or into vaginal inserts.

The injectable formulation may be a suspension and can be prepared by reducing the active agent to a very fine powder with a ball mill, micronizer, colloid mill, or other appropriate equipment and then suspending the material in a liquid in which it is insoluble.

Alternatively the injection can be packaged as a dry solid rather than in conjunction with a solvent or vehicle due to the instability of the therapeutic agent in the presence of

the liquid component, and that upon addition of suitable vehicle(s), yield solutions or suspensions conforming in all respects to the requirements for injection.

In other forms the active ingredient is packaged as a dry powder in conjunction with the liquid material for use at the time of reconstitution.

A suitable injectable formulation, particularly where mixed from crystals of trientine dihydrochloride, sterile water, a phosphate buffering system (eg to pH 7.4 +/- 0.1) and optionally any other desirable excipients, diluent, adjuvants, all as indicated in the art as useful in parenteral formulations. For example a preferred method of preparing a formulation for parenteral administration may be prepared by dissolution of trientine hydrochloride in water and dilution out to 154mM in phosphate buffered saline.

Whilst other buffering systems may be used preferably all formulations are aqueous based.

DATED THIS 17 DAY OF March 2003
AJ PARK
PER
AGENTS FOR THE APPLICANT

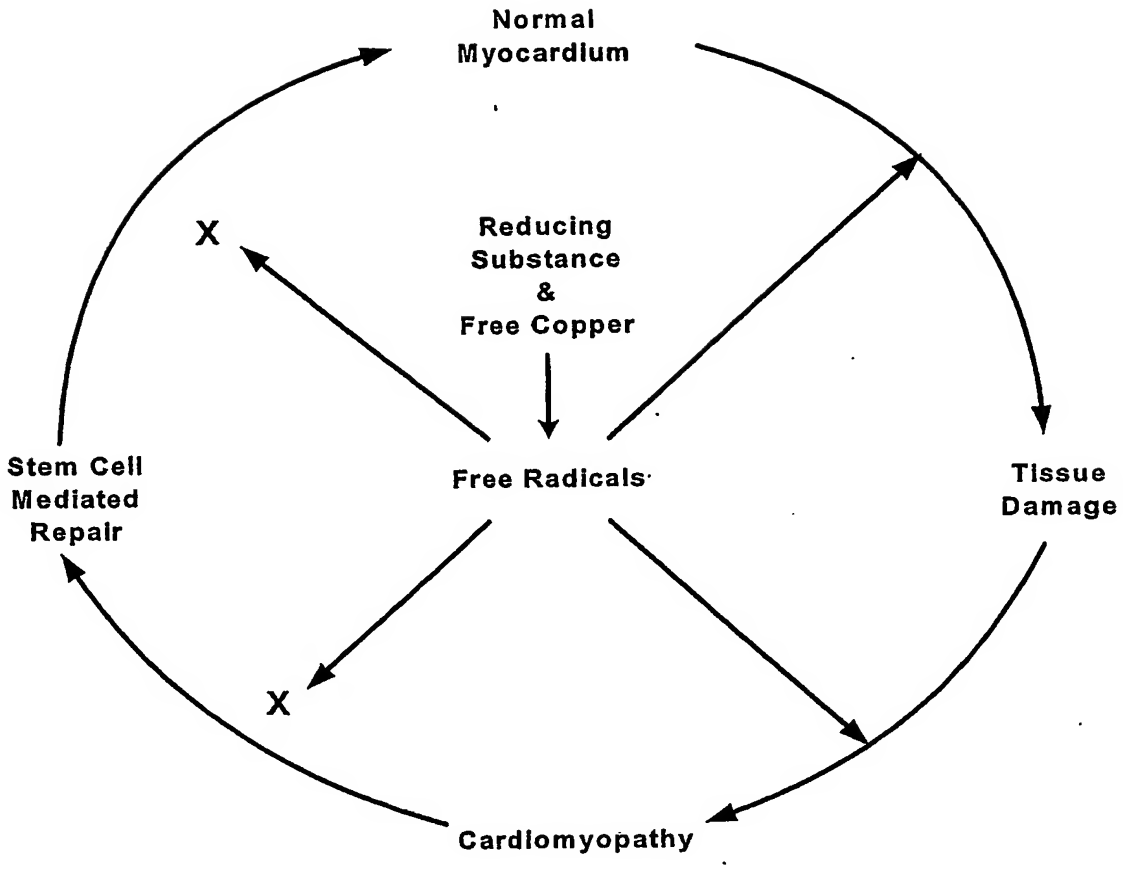

Normal
Myocardium
X
Reducing
Substance
&
Free Copper
Stem Cell
Mediated
Repair
Free Radicals
Tissue
Damage
X
Cardiomyopathy

FIGURE 1

Hypothesis

1. **Glyco-oxidation product ion (AGE* formation)**

   Lipid or Protein + glucose $\rightarrow$ {Protein - AGE
   {Lipid - AGE

   Cu-AGE Protein or Cu-AGE Lipid $\rightarrow$ Protein + $O_2^{\cdot -}$

2. **Superoxide dismutase**

   $2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$

3. **Haber-Weiss Reaction** (Copper salt catalyst)

   $H_2O_2 + O_2^{\cdot -} \rightarrow O_2 + OH\bullet + OH^-$

*AGE = Advance Glycation Endproduct

FIGURE 2

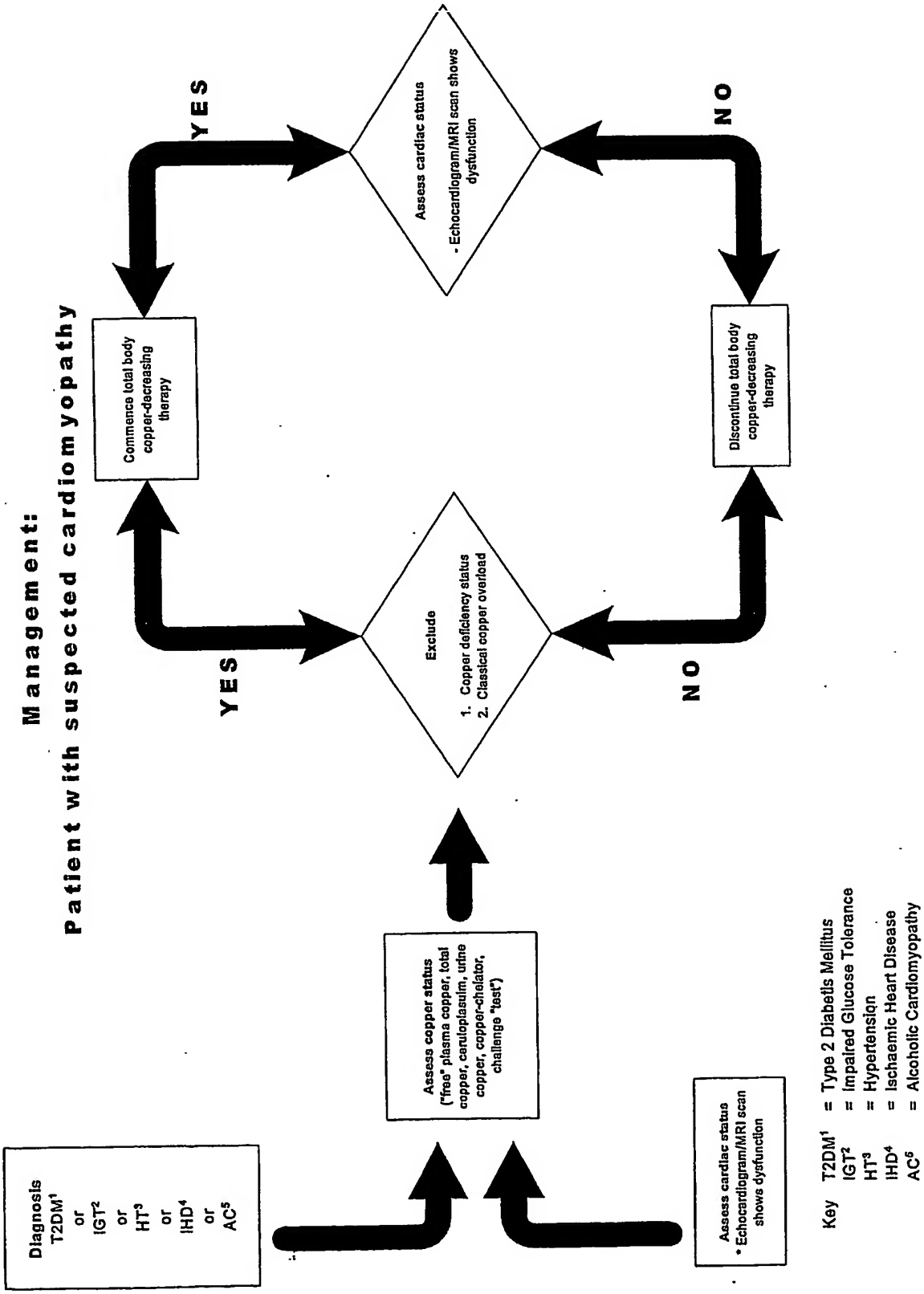
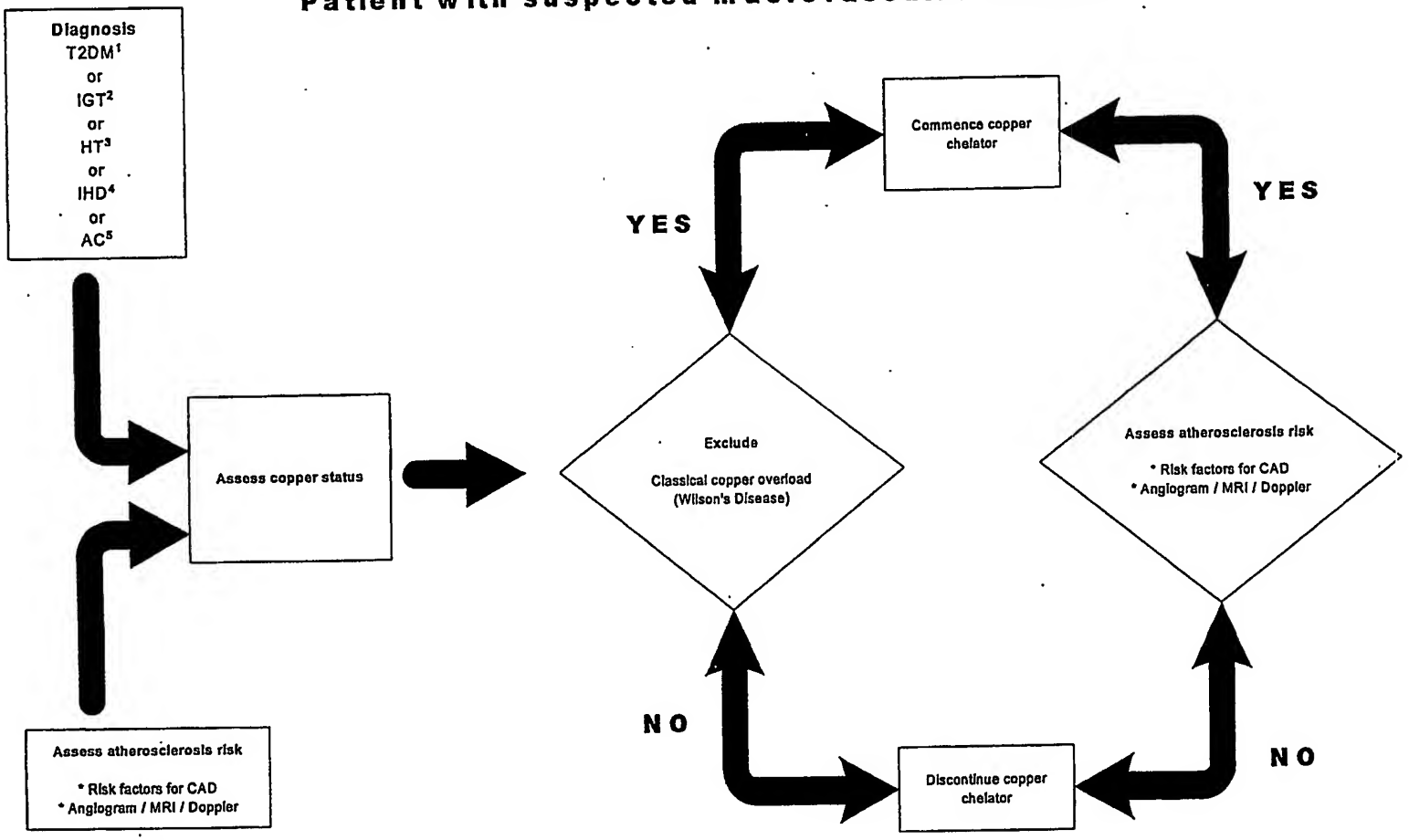

Management:
Patient with suspected cardiomyopathy
Diagnosis
T2DM1
or
IGT2
or
HT3
or
IHD4
or
AC5
Assess cardiac status
• Echocardiogram/MRI scan shows dysfunction
Assess copper status
("free" plasma copper, total copper, ceruloplasmin, urine copper, copper-chelator, challenge "test")
Exclude
1. Copper deficiency status
2. Classical copper overload
YES
NO
Assess cardiac status
- Echocardiogram/MRI scan shows dysfunction
YES
NO
Commence total body copper-decreasing therapy
Discontinue total body copper-decreasing therapy
Key T2DM1 = Type 2 Diabetis Mellitus
IGT2 = Impaired Glucose Tolerance
HT3 = Hypertension
IHD4 = Ischaemic Heart Disease
AC5 = Alcoholic Cardiomyopathy

FIGURE 3

FIGURE 4

Management:

Patient with suspected macrovascular disease



Key: T2DM1 = Type 2 Diabetis Mellitus
IGT2 = Impaired Glucose Tolerance
HT3 = Hypertension
IHD4 = Ischaemic Heart Disease
AC5 = Alcoholic Cardiomyopathy

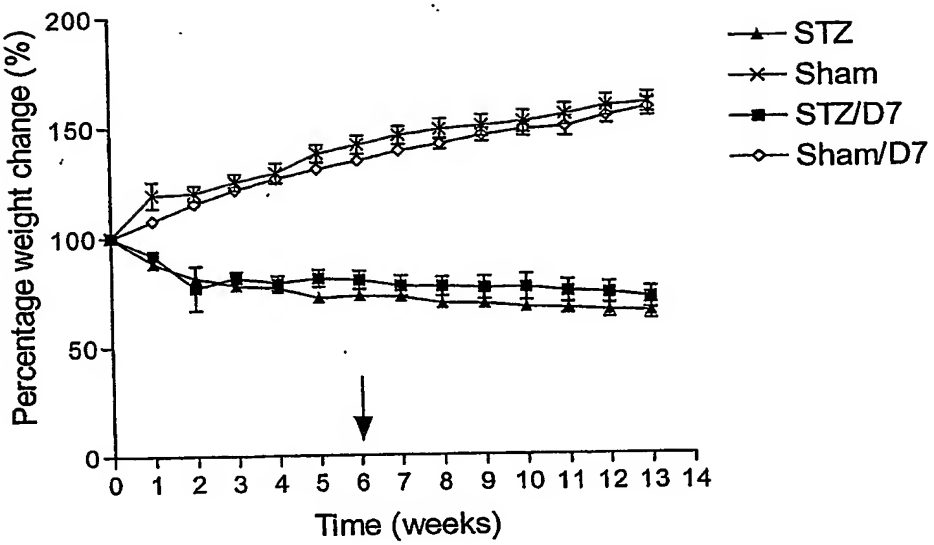

Percentage weight change (%)
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (weeks)
STZ
Sham
STZ/D7
Sham/D7

FIGURE 5

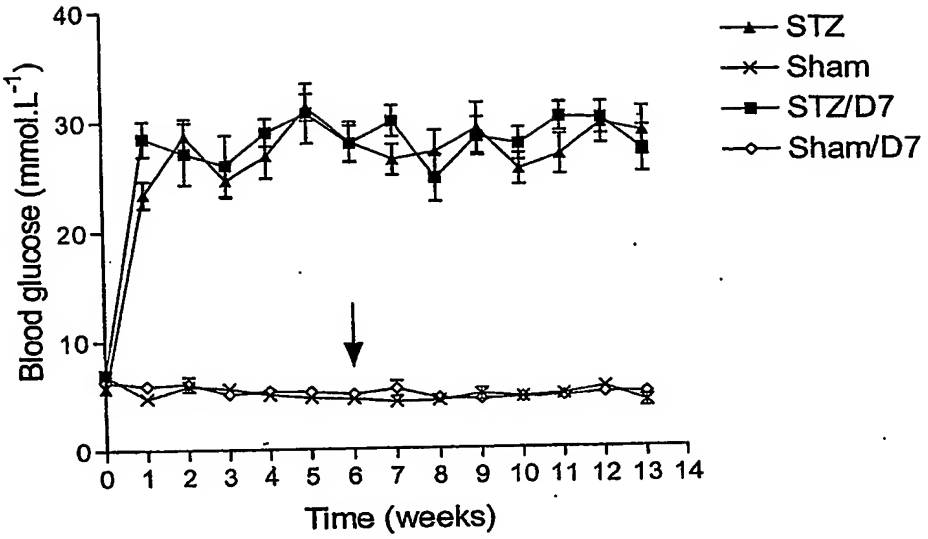
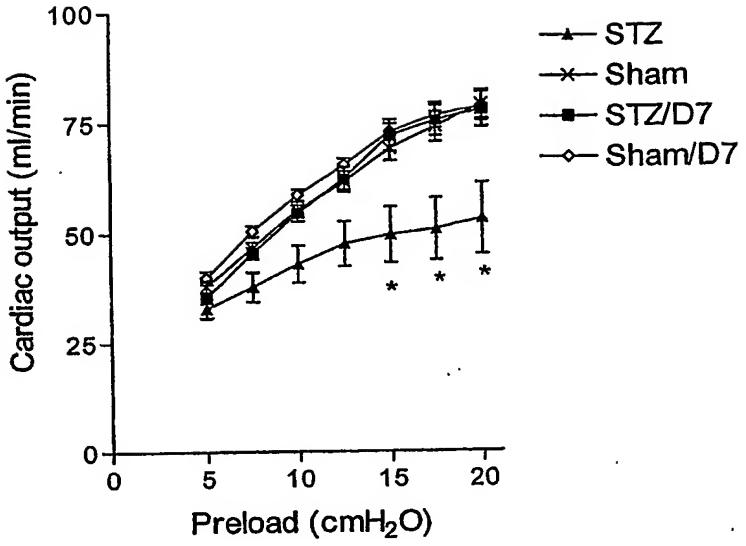
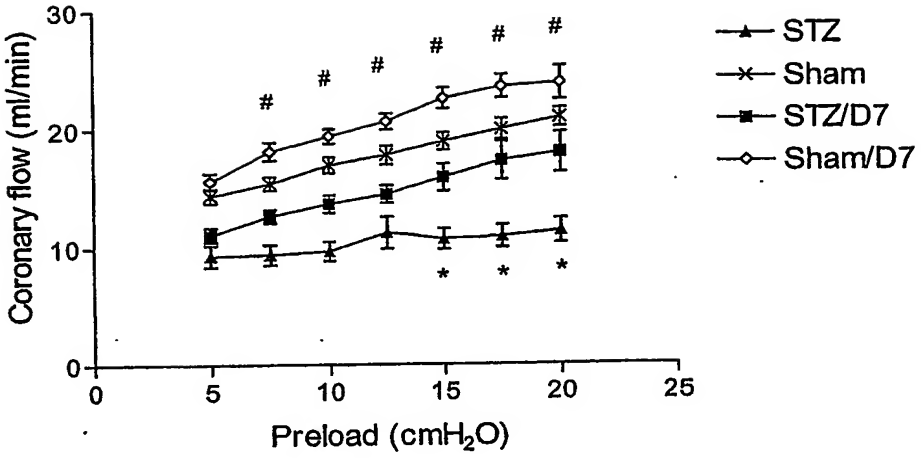

40
30
20
10
0
Blood glucose (mmol.L-1)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (weeks)
STZ
Sham
STZ/D7
Sham/D7

FIGURE 6



* p<0.05: STZ v STZ/D7

FIGURE 7



* p<0.05: STZ v STZ/D7, #.p<0.05: STZ/D7 v Sham/D7.

FIGURE 8

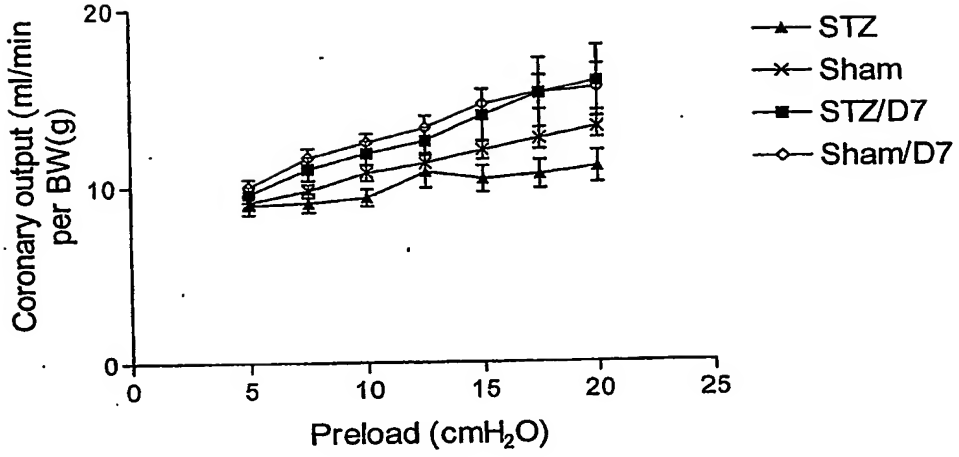
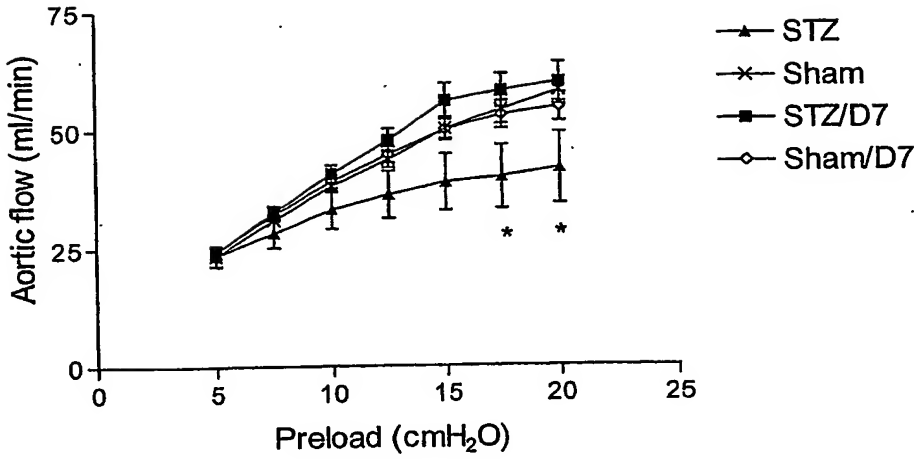
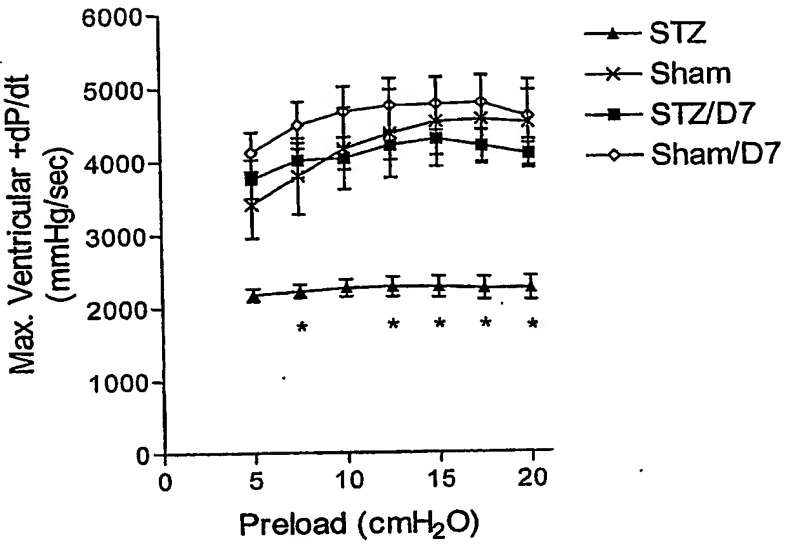

Coronary output (ml/min per BW(g)
20
10
0
0
5
10
15
20
25
Preload (cmH2O)
STZ
Sham
STZ/D7
Sham/D7

FIGURE 9



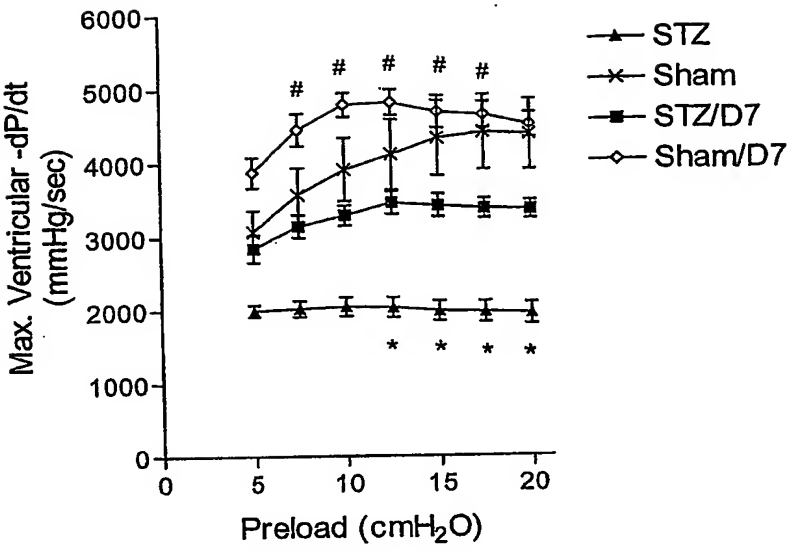
* p<0.05: STZ v STZ/D7

FIGURE 10



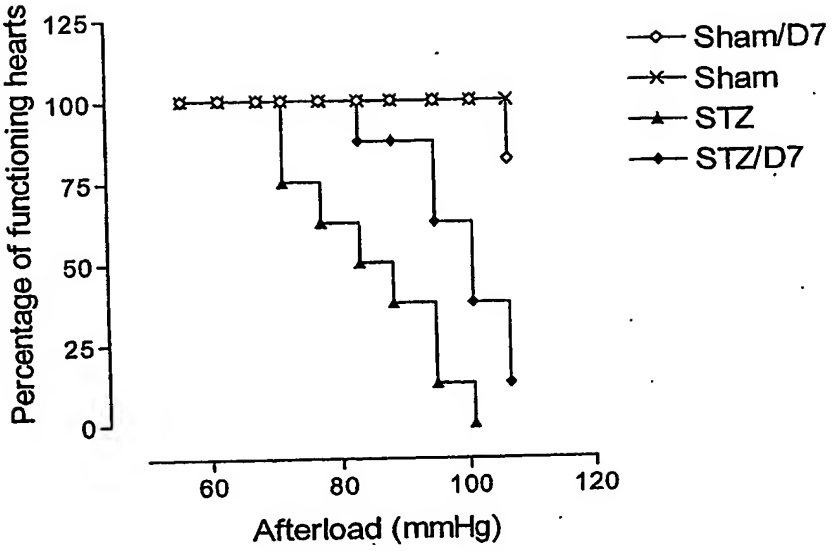
p<0.05: STZ v STZ/D7

FIGURE 11



p<0.05: STZ v STZ/D7, #.p<0.05: STZ/D7 v Sham/D7.

FIGURE 12



Wilcoxon $p<0.05$ for STZ v STZ/D7

FIGURE 13

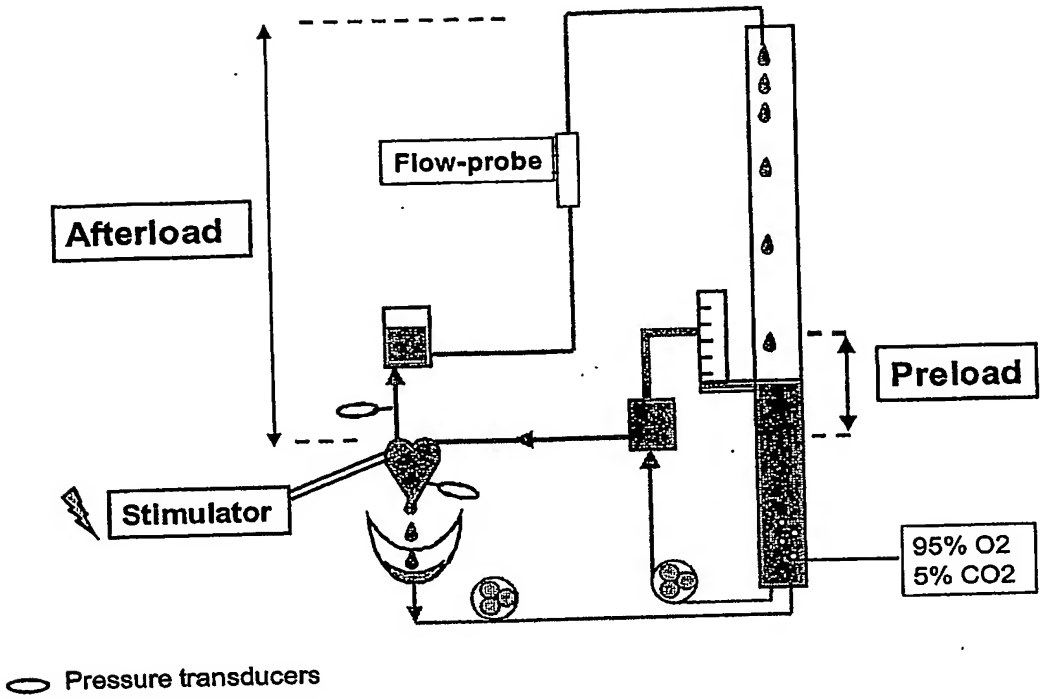

Flow-probe
Afterload
Preload
Stimulator
95% O2
5% CO2
Pressure transducers

FIGURE 14

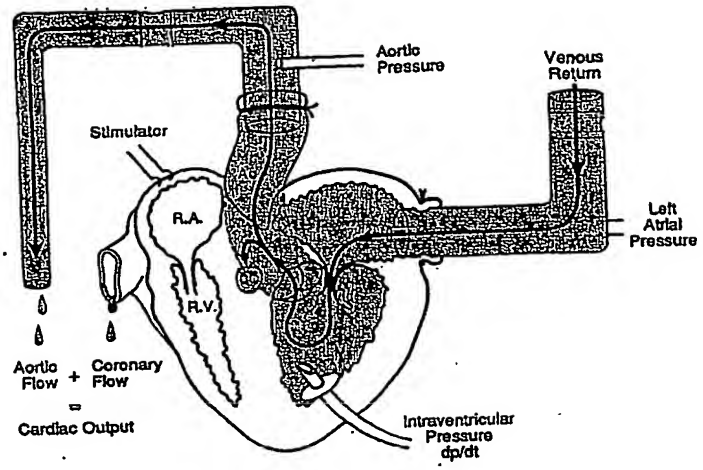
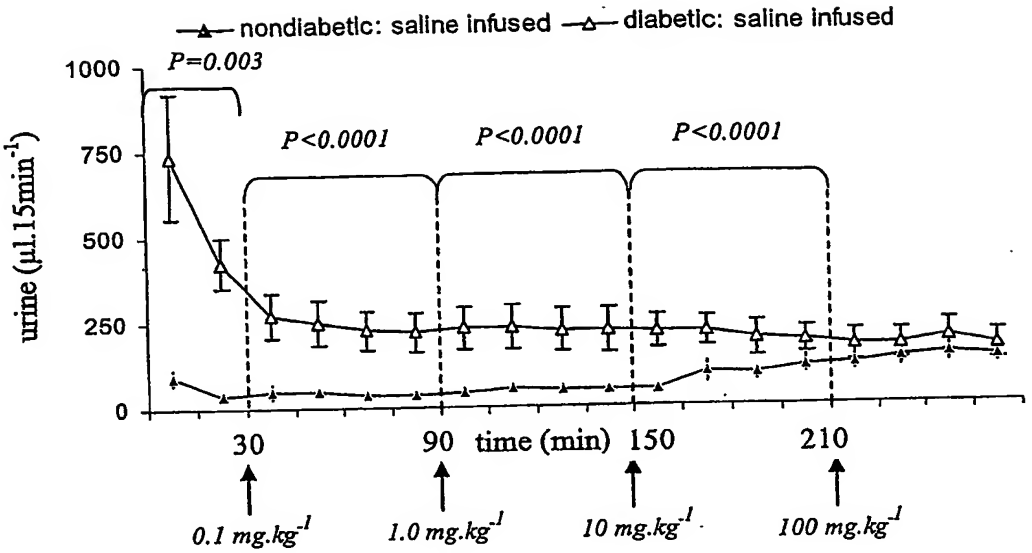

Aortic Pressure
Venous Return
Stimulator
R.A.
Left Atrial Pressure
R.V.
Aortic Flow
+
Coronary Flow
=
Cardiac Output
Intraventricular Pressure dp/dt

FIGURE 15


nondiabetic: saline infused
diabetic: saline infused
P=0.003
P<0.0001
P<0.0001
P<0.0001
urine (µl.15min-1)
1000
750
500
250
0
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

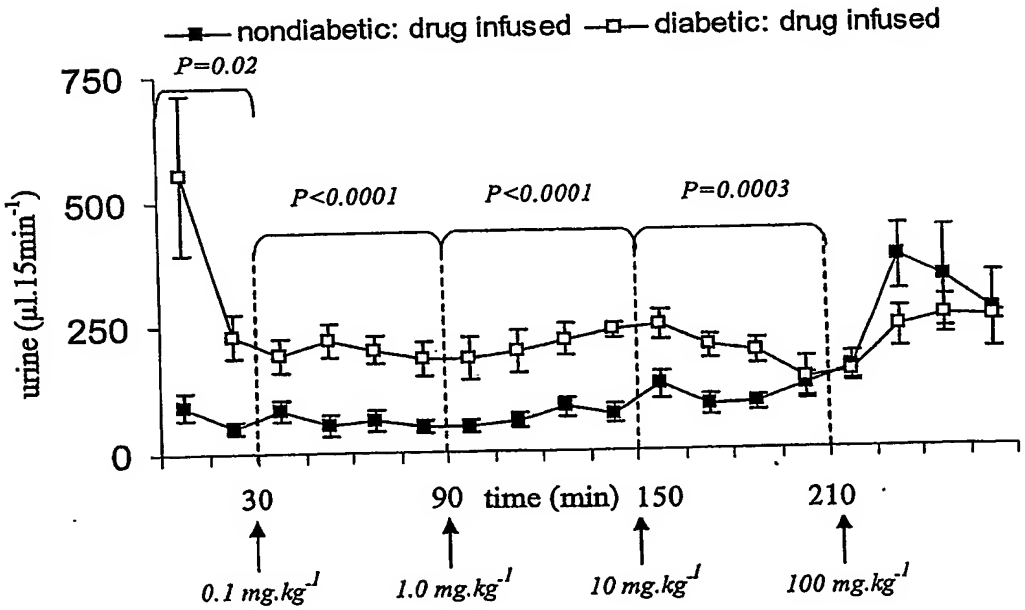

nondiabetic: drug infused
diabetic: drug infused
P=0.02
P<0.0001
P<0.0001
P=0.0003
urine (µl.15min-1)
750
500
250
0
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 16

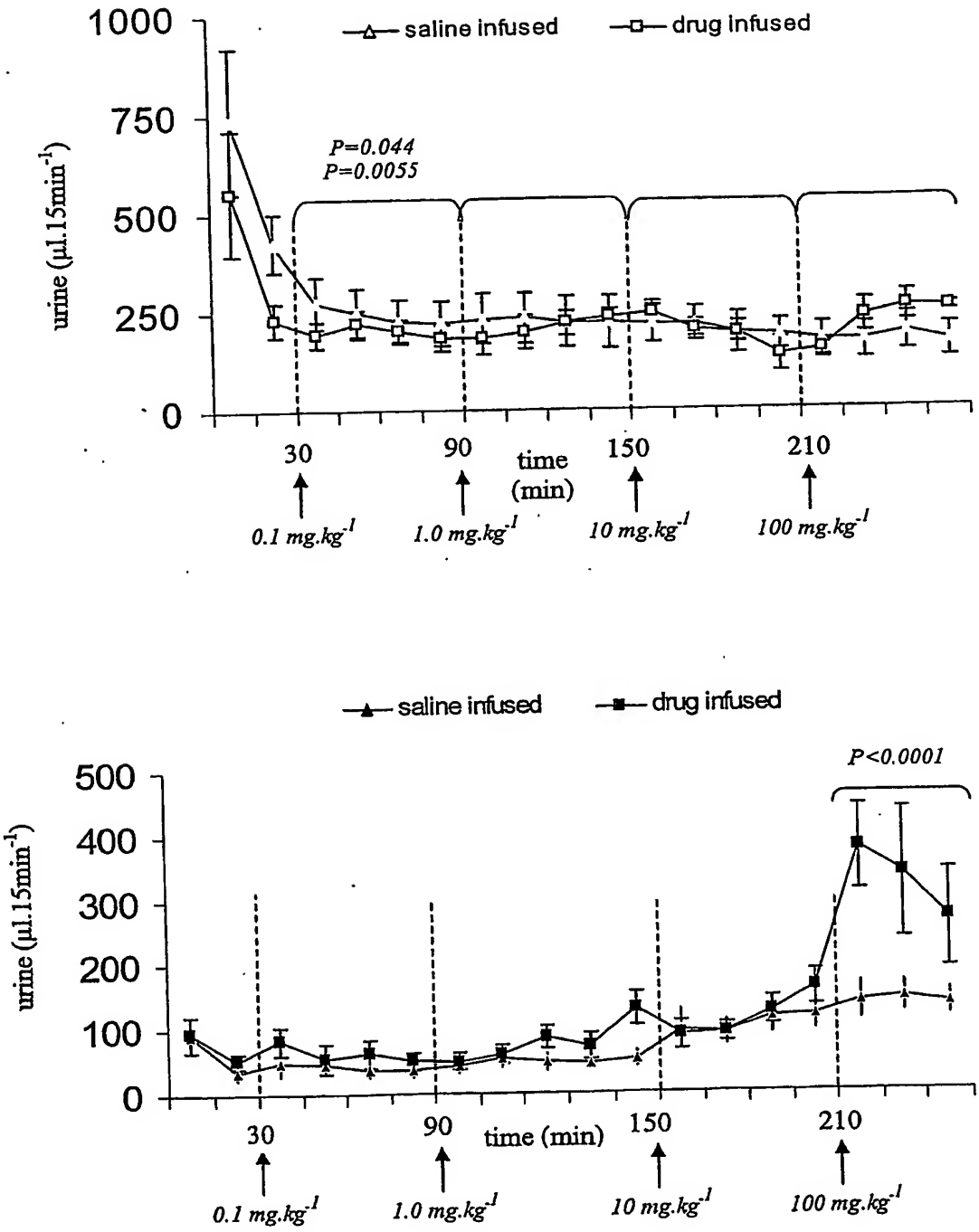
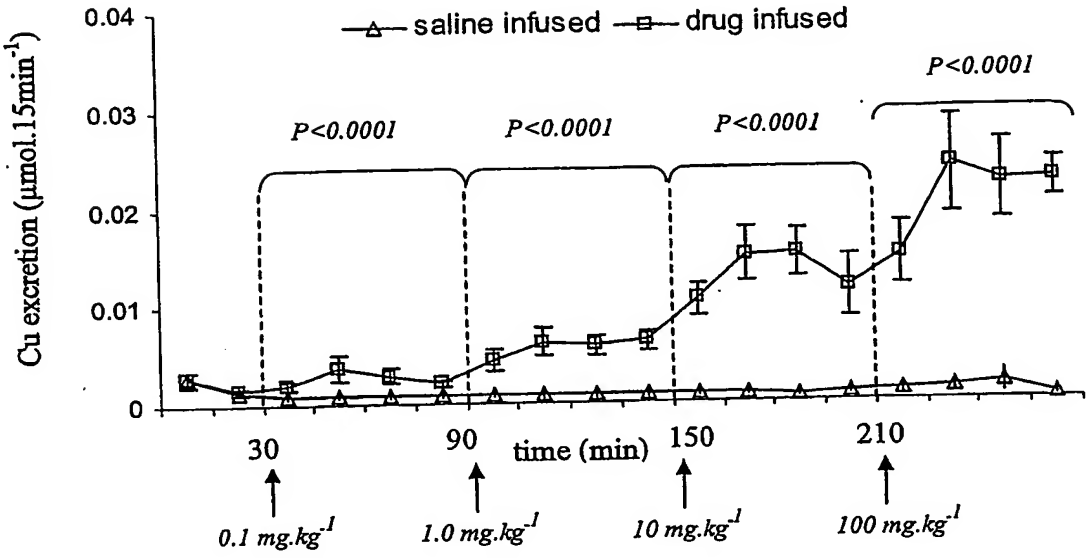

saline infused
drug infused
1000
750
500
250
0
urine (µl.15min-1)
P=0.044
P=0.0055
30
90
150
210
time (min)
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1
saline infused
drug infused
P<0.0001
500
400
300
200
100
0
urine (µl.15min-1)
30
90
150
210
time (min)
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 17


saline infused
drug infused
Cu excretion (µmol.15min-1)
0.04
0.03
0.02
0.01
0
P<0.0001
P<0.0001
P<0.0001
P<0.0001
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

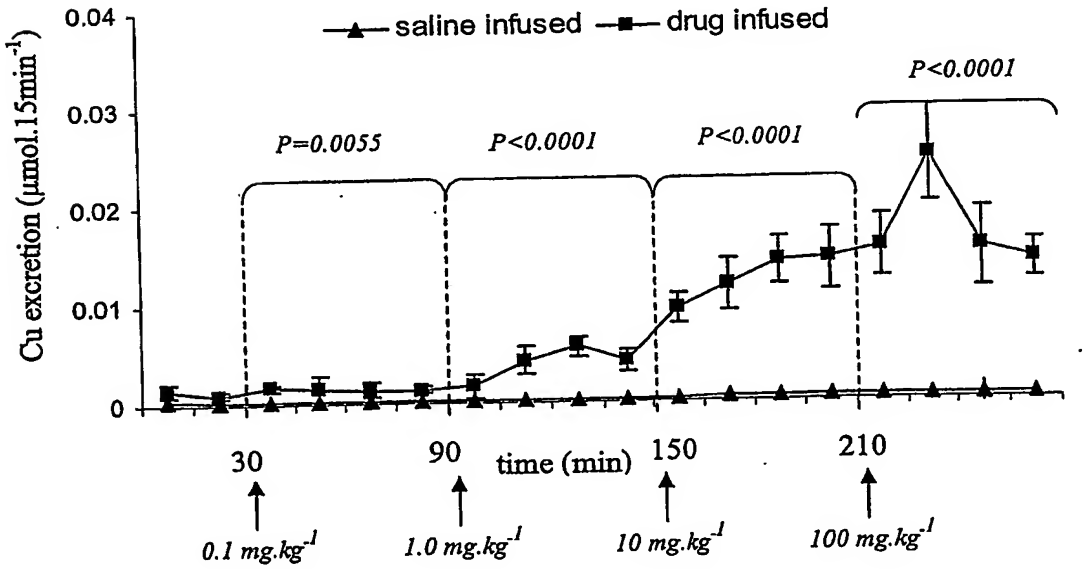

saline infused
drug infused
Cu excretion (µmol.15min-1)
0.04
0.03
0.02
0.01
0
P=0.0055
P<0.0001
P<0.0001
P<0.0001
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 18

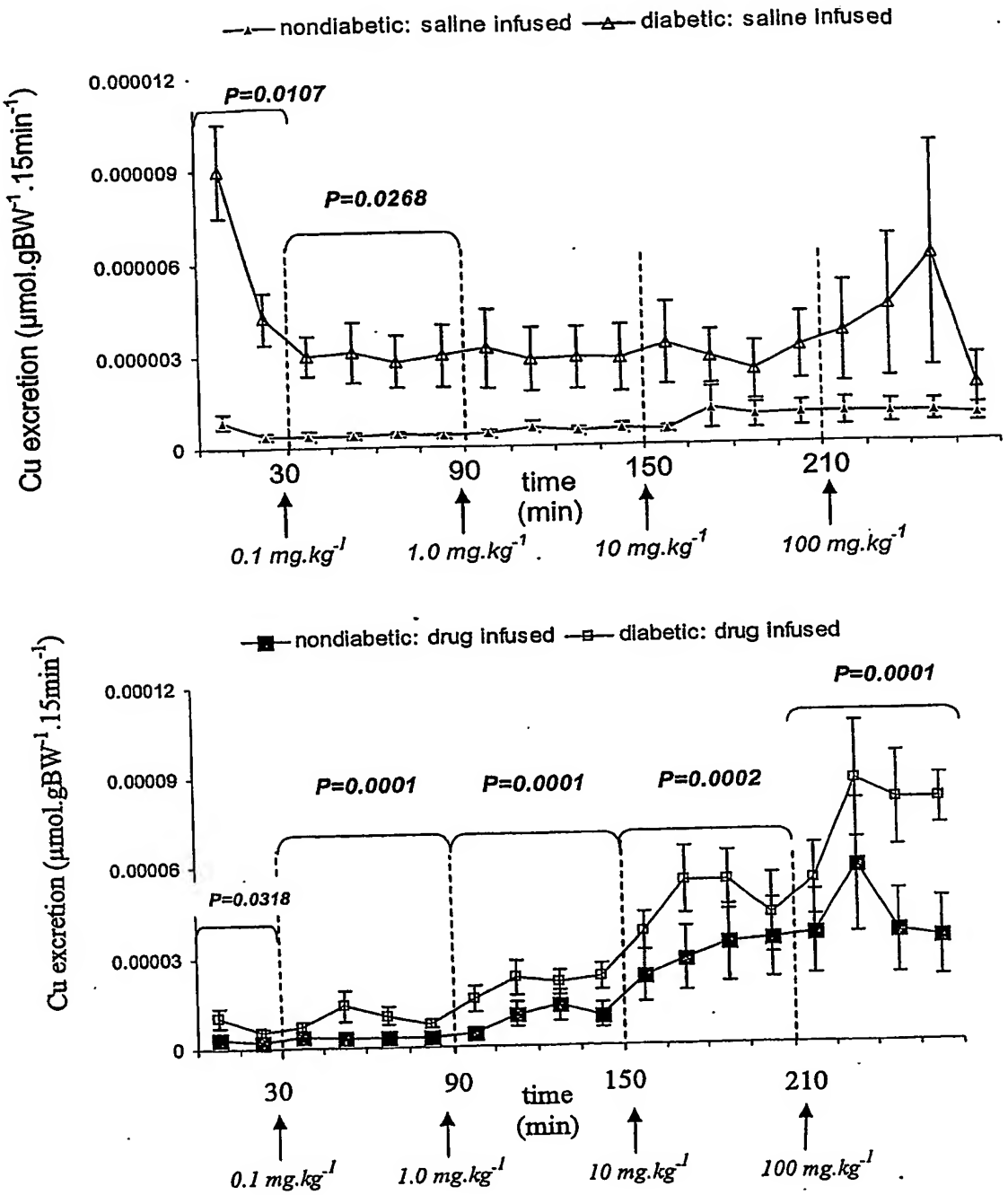

nondiabetic: saline infused
diabetic: saline infused
Cu excretion (µmol.gBW-1.15min-1)
0.000012
0.000009
0.000006
0.000003
0
P=0.0107
P=0.0268
30
90
150
210
time (min)
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1
nondiabetic: drug infused
diabetic: drug infused
Cu excretion (µmol.gBW-1.15min-1)
0.00012
0.00009
0.00006
0.00003
0
P=0.0318
P=0.0001
P=0.0001
P=0.0002
P=0.0001
30
90
150
210
time (min)
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 19

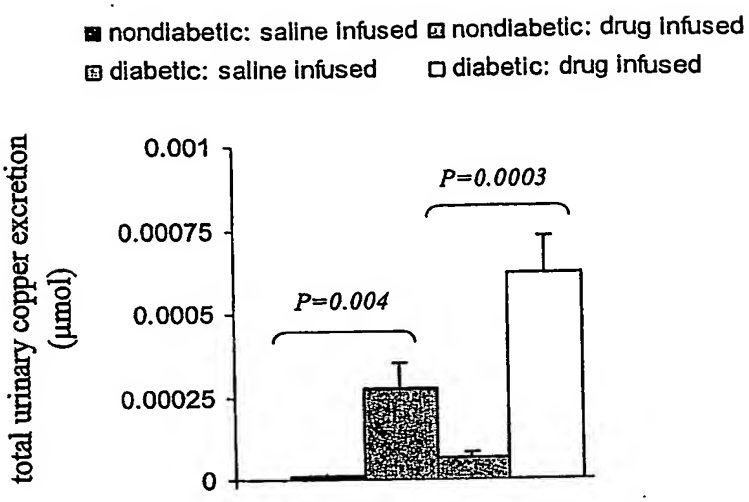

nondiabetic: saline infused
nondiabetic: drug infused
diabetic: saline infused
diabetic: drug infused
total urinary copper excretion (µmol)
0.001
0.00075
0.0005
0.00025
0
P=0.004
P=0.0003

FIGURE 20

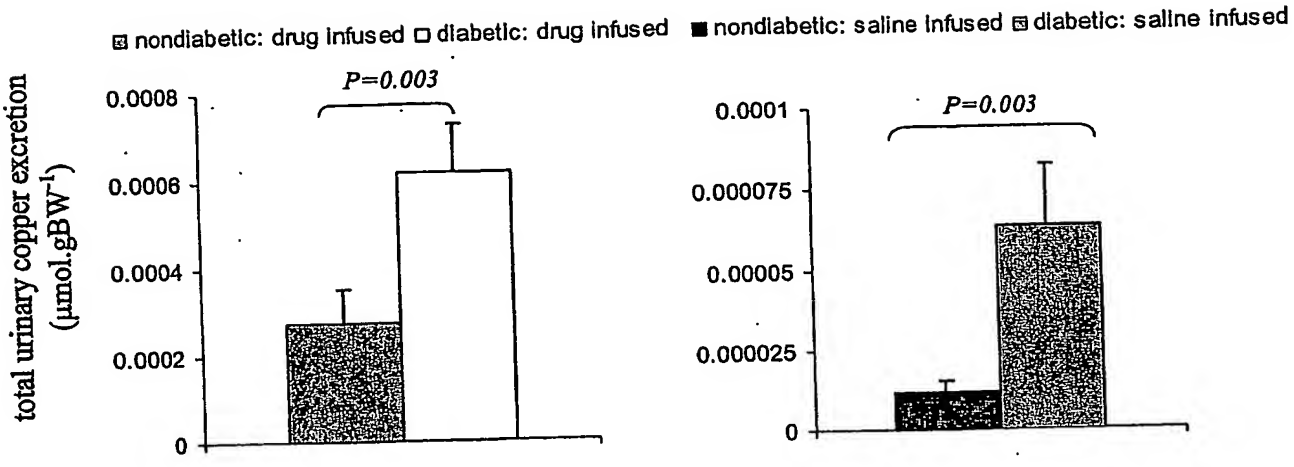

nondiabetic: drug infused
diabetic: drug infused
nondiabetic: saline infused
diabetic: saline infused
total urinary copper excretion (µmol.gBW-1)
P=0.003
0.0008
0.0006
0.0004
0.0002
0
P=0.003
0.0001
0.000075
0.00005
0.000025
0

FIGURE 21

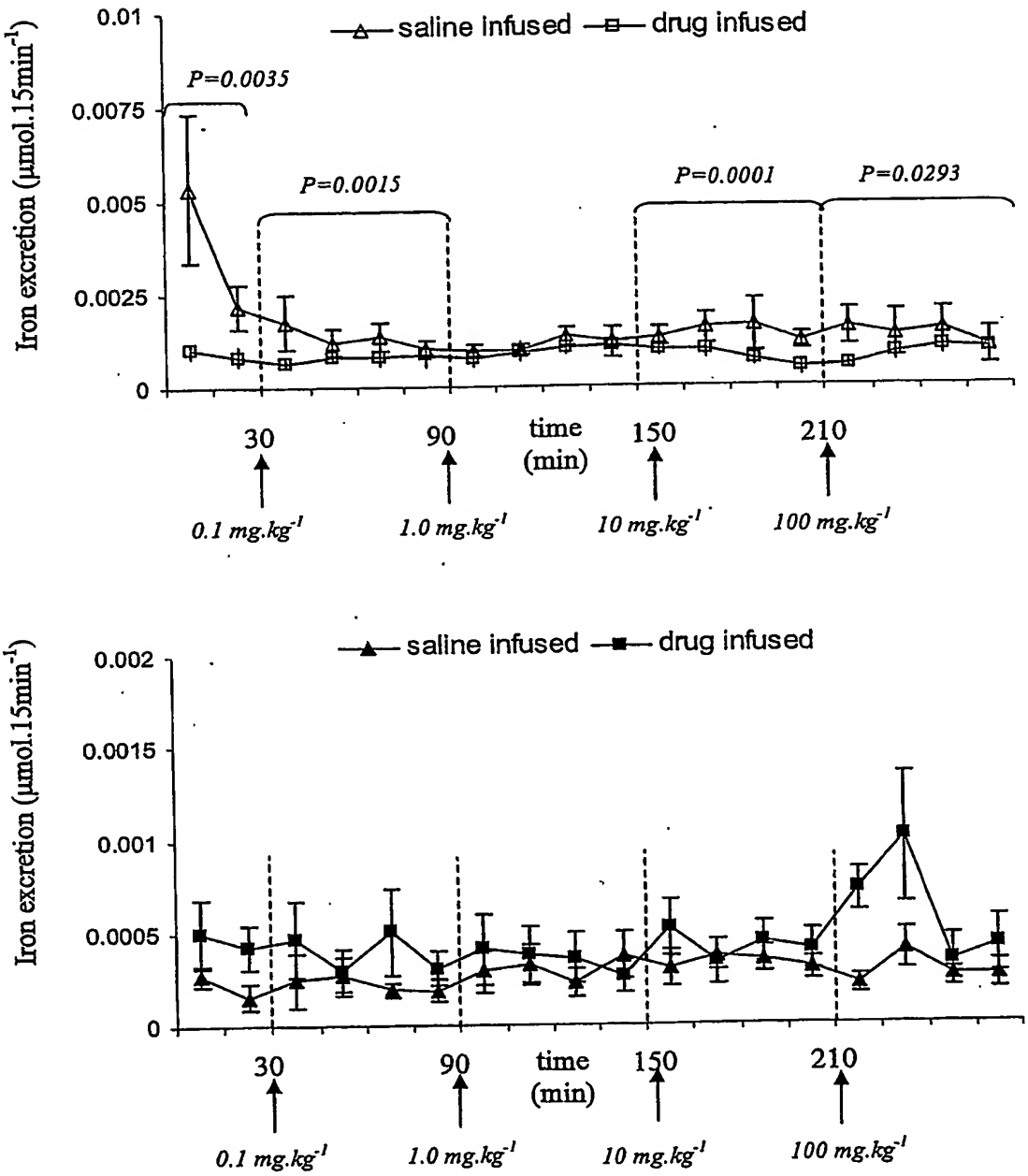

saline infused
drug infused
Iron excretion (µmol.15min-1)
0.01
0.0075
0.005
0.0025
0
P=0.0035
P=0.0015
P=0.0001
P=0.0293
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1
saline infused
drug infused
Iron excretion (µmol.15min-1)
0.002
0.0015
0.001
0.0005
0
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 22

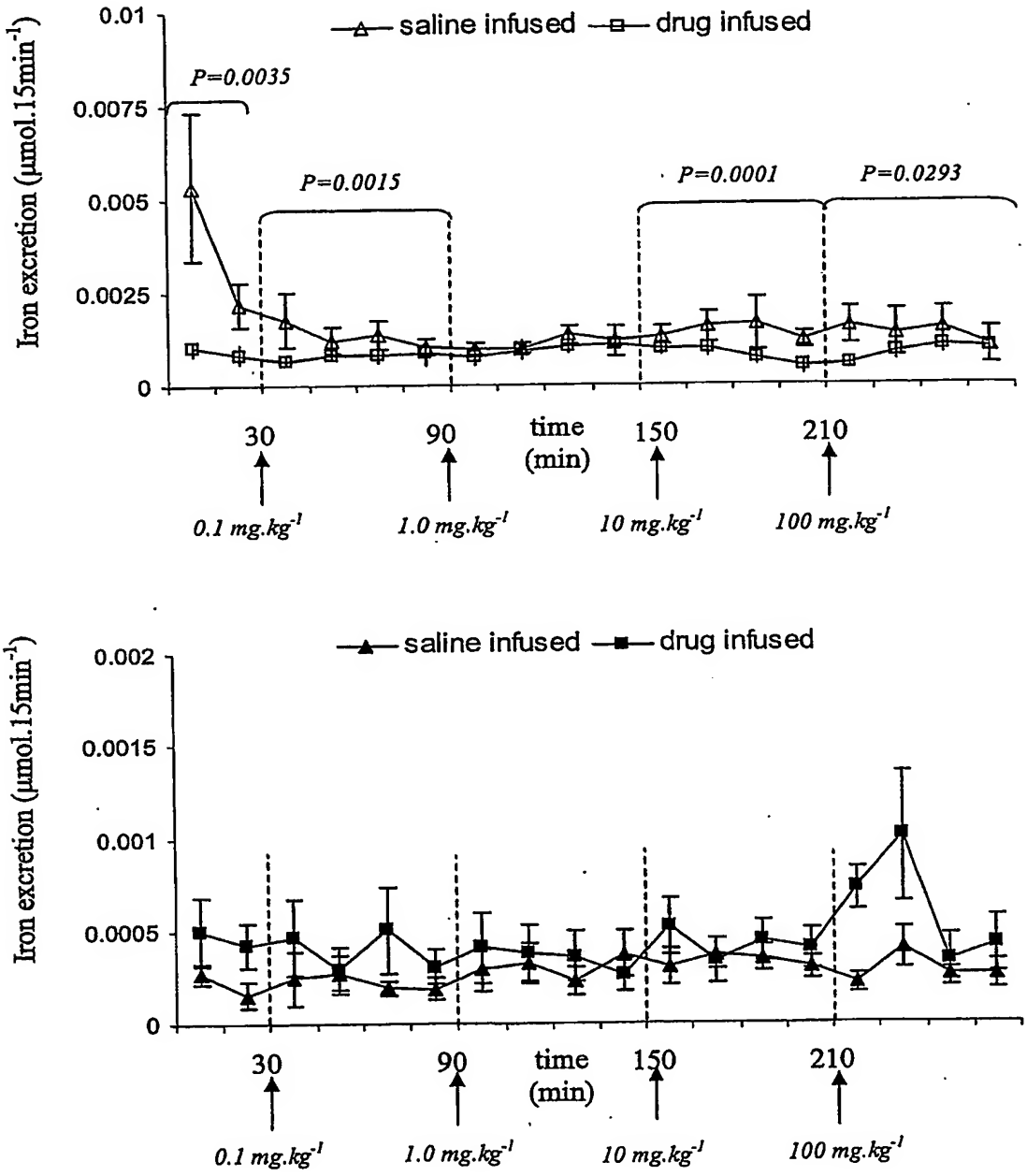

saline infused
drug infused
Iron excretion (µmol.15min-1)
0.01
0.0075
0.005
0.0025
0
P=0.0035
P=0.0015
P=0.0001
P=0.0293
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1
saline infused
drug infused
Iron excretion (µmol.15min-1)
0.002
0.0015
0.001
0.0005
0
30
90
time (min)
150
210
0.1 mg.kg-1
1.0 mg.kg-1
10 mg.kg-1
100 mg.kg-1

FIGURE 23

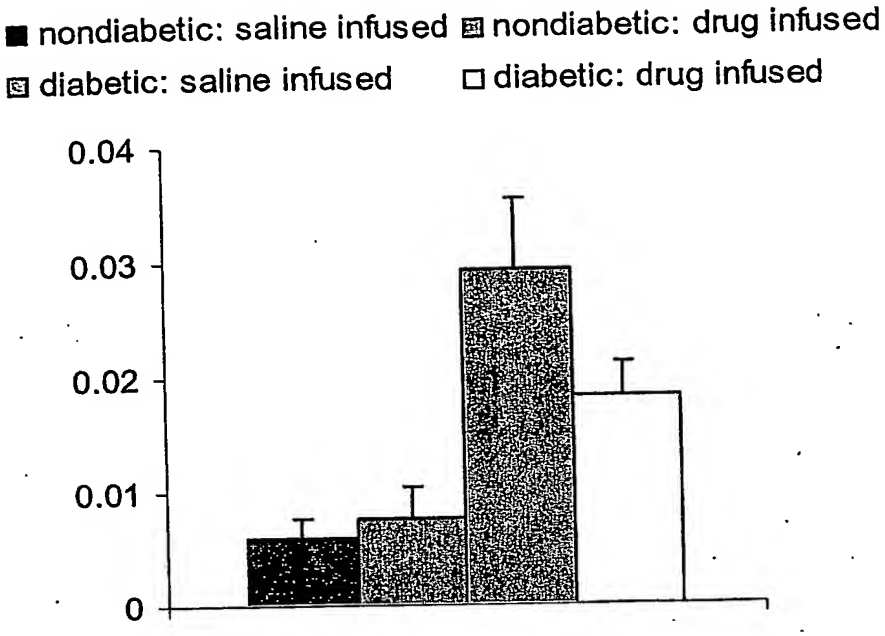

nondiabetic: saline infused
nondiabetic: drug infused
diabetic: saline infused
diabetic: drug infused
0.04
0.03
0.02
0.01
0

FIGURE 24

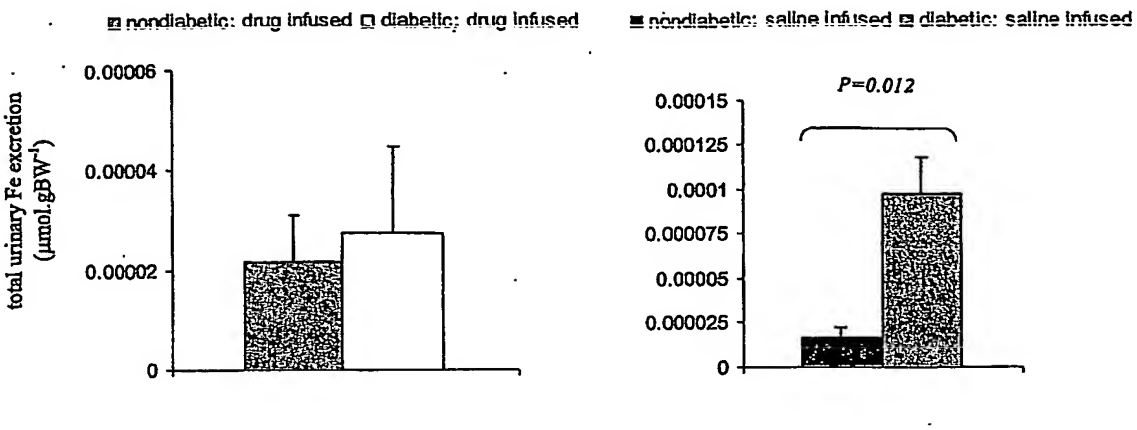

nondiabetic: drug infused
diabetic: drug infused
nondiabetic: saline infused
diabetic: saline infused
total urinary Fe excretion (µmol.gBW⁻¹)
0.00006
0.00004
0.00002
0
P=0.012
0.00015
0.000125
0.0001
0.000075
0.00005
0.000025
0

FIGURE 25

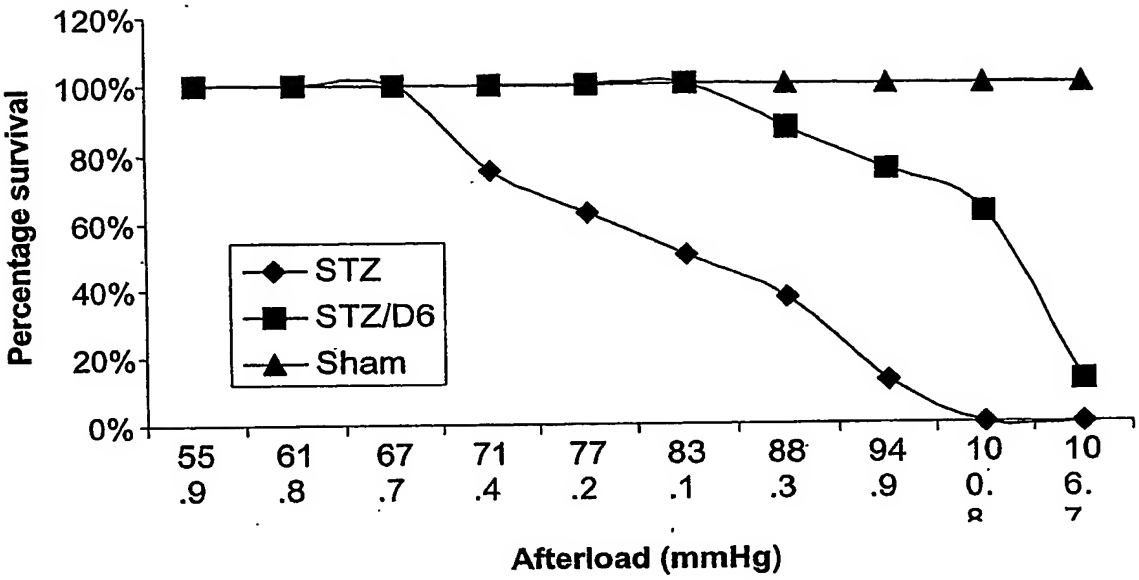

120%
100%
80%
60%
40%
20%
0%
Percentage survival
STZ
STZ/D6
Sham
55 .9
61 .8
67 .7
71 .4
77 .2
83 .1
88 .3
94 .9
10 0. 8
10 6. 7
Afterload (mmHg)

FIGURE 26

Cu excretion			Dose level		
Mixed Model Effects	**Baseline**	**0.1 mg.kg^{-1}**	**1.0 mg.kg^{-1}**	**10 mg.kg^{-1}**	**100 mg.kg^{-1}**
Diabetes	$F_{1,24}$ = 18.52	$F_{1,24}$ = 19.82	$F_{1,24}$ = 21.92	$F_{1,24}$ = 9.93	$F_{1,24}$ = 17.32
(normal/diabetic rats)	P = 0.0002	P = 0.0002	P < 0.0001	P < 0.0001	P < 0.0003
Drug	$F_{1,24}$ = 1.73	$F_{1,24}$ = 24.94	$F_{1,24}$ = 78.36	$F_{1,24}$ = 135.36	$F_{1,24}$ = 162.17
(drug/saline)	*NS*	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
Interaction	$F_{1,24}$ = 0.16	$F_{1,24}$ = 3.58	$F_{1,24}$ = 7.16	$F_{1,24}$ = 6.02	$F_{1,24}$ = 12.43
	NS	*NS*	P < 0.0132	P < 0.0218	P < 0.0017
Sampling time (repeated measure)	t_1, t_2	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4

Fe excretion			Dose level		
Mixed Model Effects	**Baseline**	**0.1 mg.kg^{-1}**	**1.0 mg.kg^{-1}**	**10 mg.kg^{-1}**	**100 mg.kg^{-1}**
Diabetes	$F_{1,23}$ = 12.87	$F_{1,23}$ = 15.82	$F_{1,24}$ = 22.68	$F_{1,24}$ = 14.93	$F_{1,24}$ = 7.35
(normal/diabetic rats)	P = 0.0016	P = 0.0006	P < 0.0001	P = 0.0007	P = 0.0122
Drug	$F_{1,23}$ = 8.6	$F_{1,23}$ = 7.89	$F_{1,24}$ = 12.23	$F_{1,24}$ = 10.91	$F_{1,24}$ = 2.47
(drug/saline)	P = 0.0075	P = 0.01	P < 0.0019	P = 0.003	P = 0.1292
Interaction	$F_{1,23}$ = 12.10	$F_{1,23}$ = 15.06	$F_{1,24}$ = 14.07	$F_{1,24}$ = 17.72	$F_{1,24}$ = 16.76
	P = 0.002	P = 0.0008	P = 0.001	P = 0.0003	P = 0.0004
Sampling time (repeated measure)	2	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4	t_1, t_2, t_3, t_4

FIGURE 27

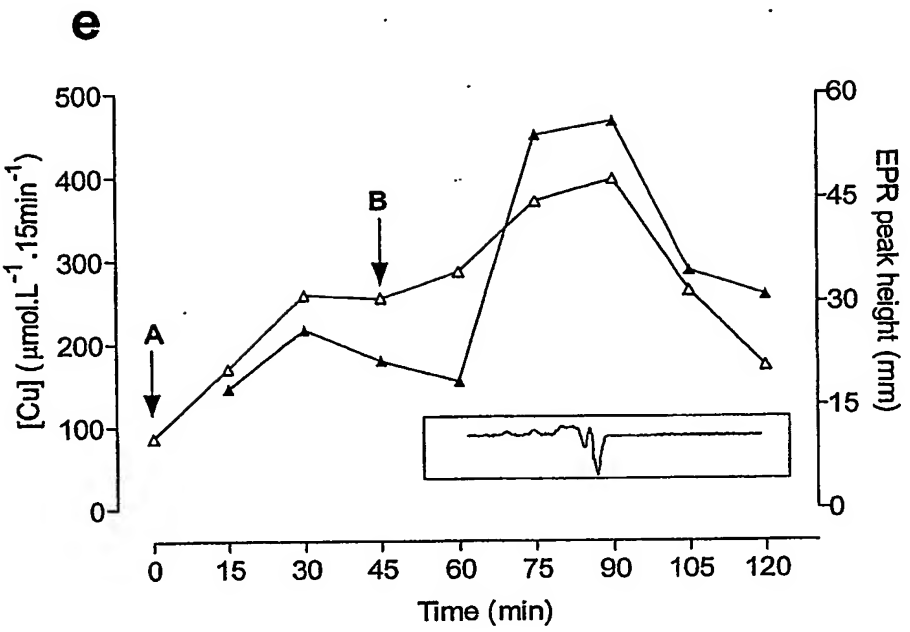

e
500
400
300
200
100
0
[Cu] (µmol.L-1.15min-1)
A
B
60
45
30
15
0
EPR peak height (mm)
0
15
30
45
60
75
90
105
120
Time (min)

FIGURE 28

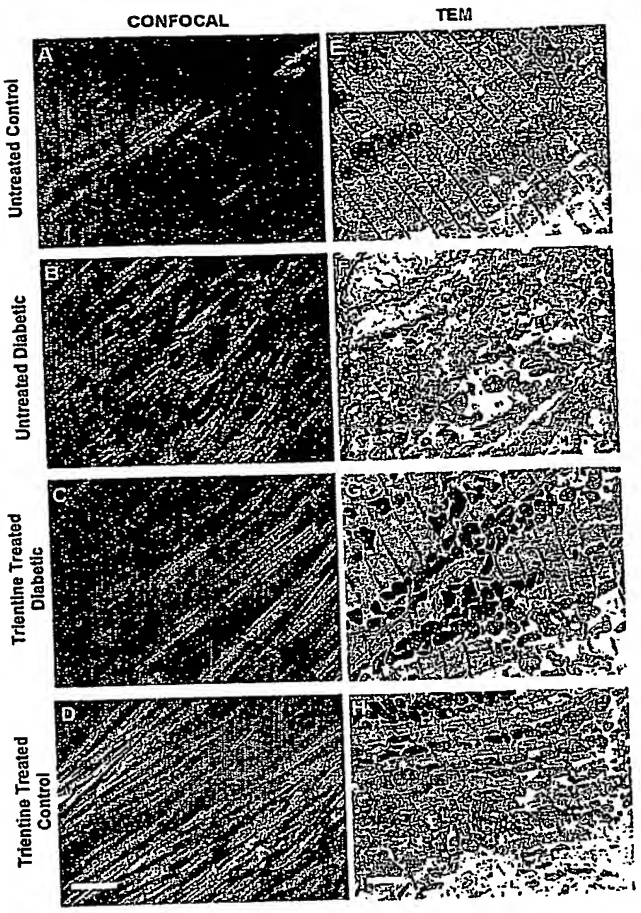

CONFOCAL
TEM
Untreated Control
Untreated Diabetic
Trientine Treated Diabetic
Trientine Treated Control
A
B
C
D
E
F
G
H

Figure 29

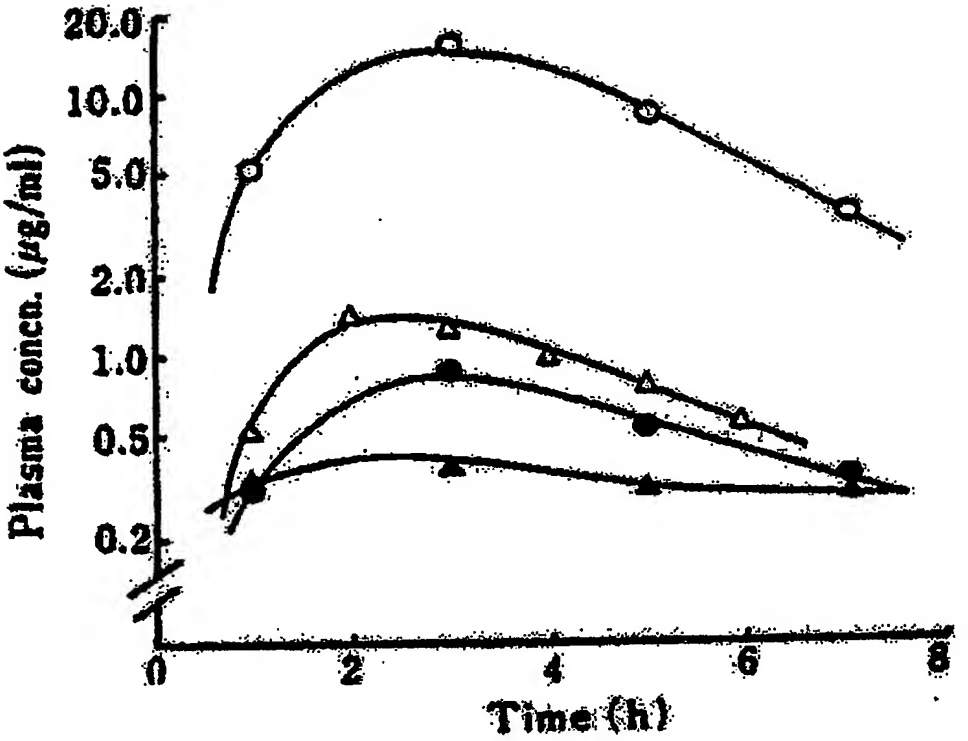


FIGURE 30

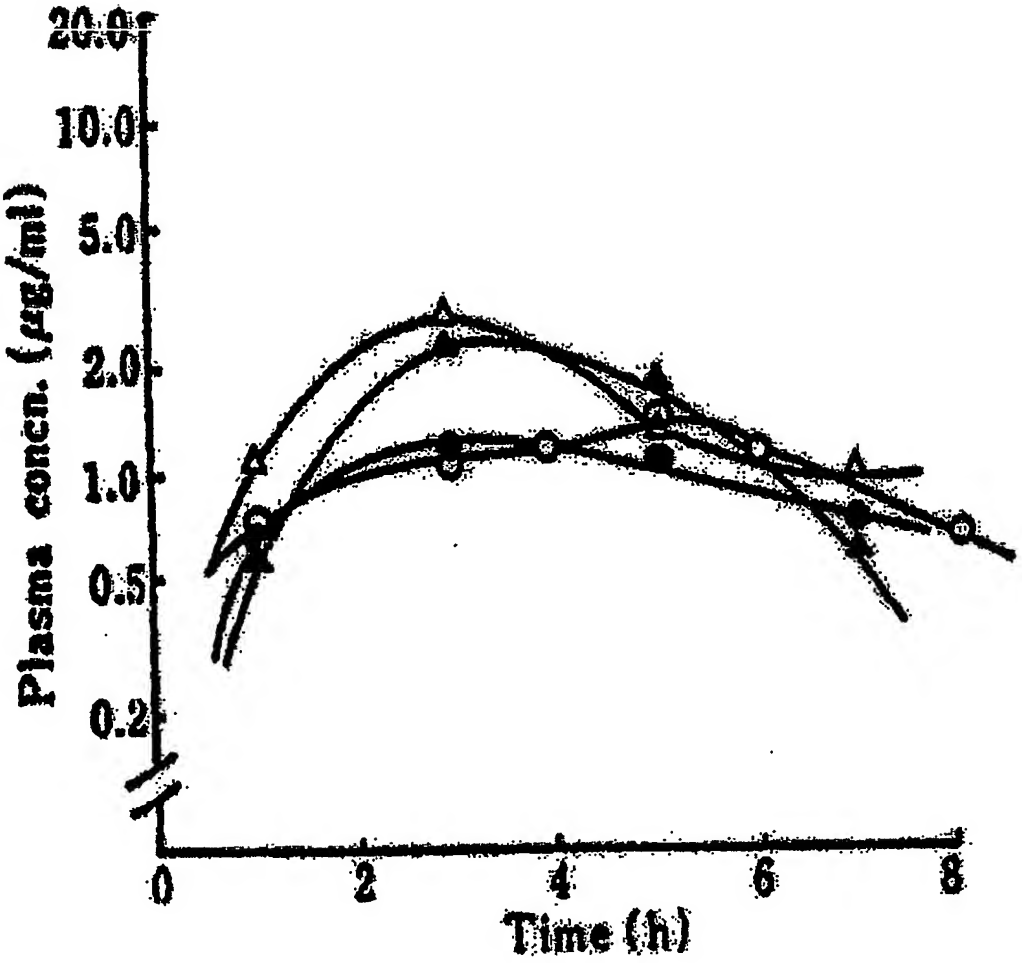


FIGURE 31

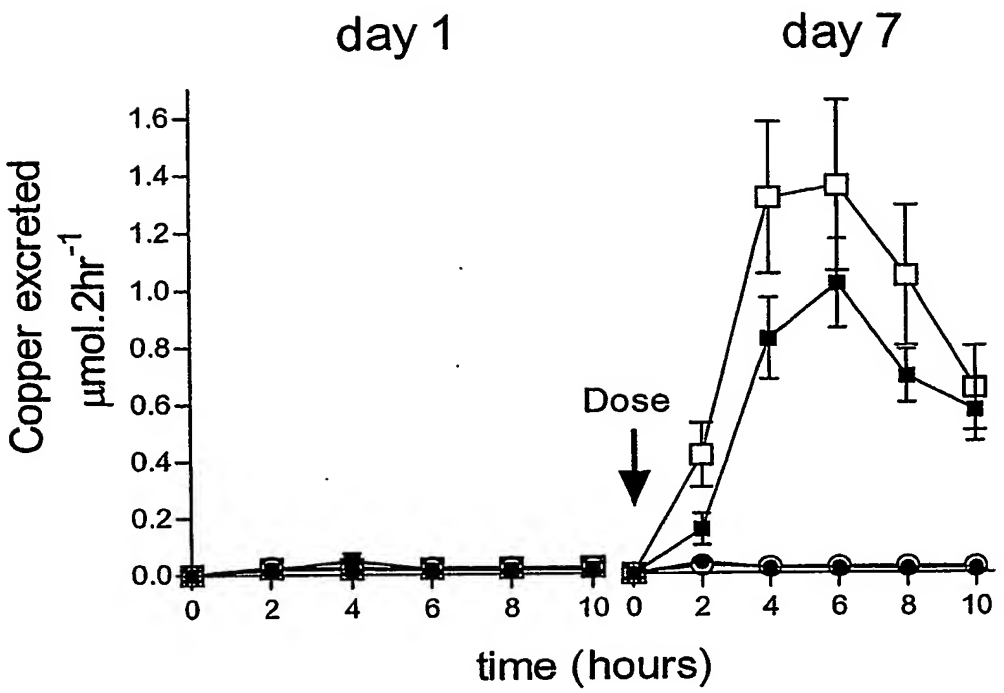

day 1
day 7
Copper excreted
µmol.2hr-1
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Dose
0
2
4
6
8
10
0
2
4
6
8
10
time (hours)

FIGURE 32

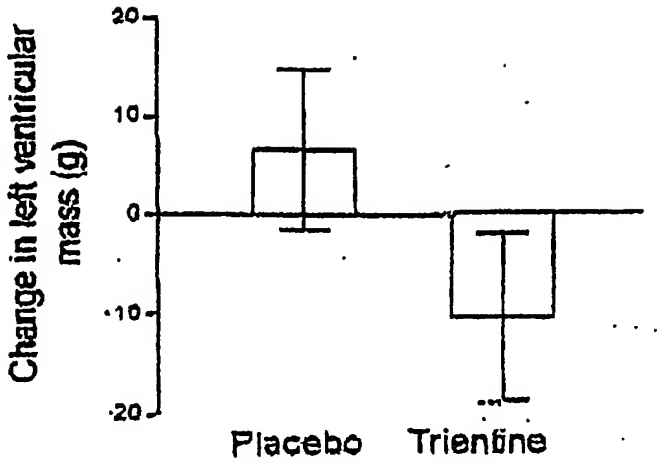

Change in left ventricular mass (g)
20
10
0
-10
-20
Placebo
Trientine

FIGURE 33